# THE INFLUENCE OF OBESITY ON ORTHODONTIC TOOTH MOVEMENT A CLINICAL STUDY

Saloom, Hayder Fadhil

*Awarding institution:*
King's College London

# THE INFLUENCE OF OBESITY ON ORTHODONTIC TOOTH MOVEMENT (A CLINICAL STUDY)

**Hayder Fadhil Saloom**
BDS, MSc.

Thesis submitted for the Degree of
**Doctor of Philosophy**

Department of Orthodontics,
Department of Mucosal and Salivary Biology
King's College London Dental Institute

**January 2017**

# Abstract

Obesity is a global public health problem, arising from the interaction between behavioural, environmental and genetic factors. The implications of obesity on orthodontic treatment included orthodontic treatment plan, less cooperation, longer duration and more health-related problems. However no data exists in relation to orthodontic tooth movement (OTM) in obese patients, from either a clinical or biochemical perspective.

The aim of this project was to study the influence of obesity on OTM by measuring (1) The rate of tooth movement and the time taken to achieve completion of tooth alignment using fixed orthodontic appliances in normal weight and obese patients; (2) The effect of obesity on orofacial pain response during the early stages of orthodontic treatment with fixed-appliances; and (3) The effect of obesity on the biochemical changes in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum with and without orthodontic treatment.

Different biomarkers were detected, including (1) Obesity-related biomarkers such as adiponectin, leptin and resistin; (2) Tissue remodelling biomarkers such as Matrix metalloproteinase-8 (MMP8), Matrix metalloproteinase-9 (MMP9) and their inhibitor (TIMP-1); (3) Bone remodelling biomarker such as Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL); and (4) Inflammation biomarker such as Myeloperoxidase (MPO) and C-reactive protein (CRP).

Two main studies were conducted in this thesis:

The first study is a cross sectional study in which UWMS, GCF and serum were collected from normal weight and obese adults (18-45 years) without orthodontic treatment. Same samples were collected from a matched number, age and gender of normal weight and obese patients have fixed orthodontic appliance with 0.019 x 0.025 – inch stainless steel archwire in upper and lower arches. The rationale behind this study is to detect the effect of obesity and/or orthodontic treatment on the levels of the selected biomarkers in different bio-fluids.

The second study is a prospective cohort study in which data were collected from 12-18 years old normal weight and obese patients at 4 time-points: (T1) prior to treatment at the normal records appointment; (T2) 1 hour following placement of the fixed appliance; (T3) 1 week following placement of the fixed appliance; and (T4) at the end of alignment

stage (0.019 x 0.025 – inch stainless steel archwire in the lower arch). The responses to orthodontic treatment were assessed in different ways: (1) Rate of tooth movement using dental study casts (T1, T3, T4); (2) Pain and discomfort using a self-reporting questionnaire (1st week); and (3) Biochemical assay of markers in UWMS, GCF and peripheral serum (T1, T2, T3, T4).

The data of this study presented that the rate of OTM was significantly higher in obese patients compared to normal weight, and obese patients needed less time to achieve tooth alignment compared to normal weight, but this was non-significant. Obese patients experience higher mean pain than normal weight patients accompanied by higher consumption of analgesics. Alongside, GCF was more likely to express biochemical changes during OTM compared to UWMS and serum, with GCF-levels of leptin; resistin, MPO and RANKL were significantly different between obese and normal weight patients and associated with observed rates of OTM.

# List of publications and presentations

## Journal articles

**1-** Hayder F. Saloom, Spyridon N. Papageorgiou, Guy H. Carpenter, Martyn T. Cobourne (2017): **Impact of obesity on orthodontic tooth movement in adolescents: a prospective clinical cohort study.** (Published in Journal of Dental Research).

**2-** Hayder F. Saloom, Martyn T. Cobourne (2016): **Periodontal parameters of a self-ligating bracket.** (Commentary article published in Journal of Orthodontics, Vol. 43, 253–254).

**3-** Hayder F. Saloom, Spyridon N. Papageorgiou, Guy H. Carpenter, Martyn T. Cobourne (2016): **The effect of obesity on orofacial pain following fixed-appliance placement: a prospective clinical cohort study in adolescents** (Submitted).

**4-** Hayder F. Saloom, Guy H. Carpenter, Martyn T. Cobourne (2016): **The effect of obesity on myeloperoxidase during orthodontic alignment in adolescents.** (In preparation).

**5-** Hayder F. Saloom, Martyn T. Cobourne, Guy H. Carpenter, (2016): **The origin of salivary adiponectin.** (In preparation).

**6-** Hayder F. Saloom, Martyn T. Cobourne, Guy H. Carpenter, (2016): **The effects of obesity on the biochemical changes in saliva, gingival crevicular fluid and serum.** (In preparation).

## Book chapter

**1-** Hayder F. Saloom, Guy H. Carpenter, (2017): **Saliva and gingival crevicular fluids-contribution to mucosal defense, edited by Lesley Bergmeier (**Submitted).

## Peer viewed conference abstracts

1- Hayder F. Saloom, Martyn T. Cobourne, Guy H. Carpenter (2015): **Does saliva contain an adiponectin-like imposter?** 47th Meeting of the Continental European Division of the International Association for Dental Research (CED-IADR), co-hosted by the Scandinavian Division (NOF) at Antalya , October 2015 (Oral presentation).

2- Hayder F. Saloom, Martyn T. Cobourne, Guy H. Carpenter (2014): **Which fluid is more representative for obesity biomarkers?** 7th Annual Congress of the International Association for Dental Research Pan European Region (IADR/PER), Dubrovnik, Croatia, September 2014 (Oral presentation).

# List of contents

# List of figures

# List of tables

# Abbreviations

| | |
|---|---|
| µl: | microliter |
| AIDS: | acquired immune deficiency syndrome |
| BMI: | body mass index |
| CI: | confidence interval |
| COX: | cyclooxygenase |
| CRP: | C-reactive protein |
| DMEM | Dulbecco's modified eagles medium |
| DNA: | deoxyribonucleic acid |
| DTT: | dithiothreitol |
| ECM: | extra cellular matrix |
| EDTA: | Ethylenediaminetetraacetic Acid |
| ELISA: | enzyme linked immuno sorbant assay |
| FITC: | fluorescein iso thiocyanate |
| GCF: | gingival crevicular fluid |
| GM-CSF: | granulocyte macrophage colony stimulating factor |
| GO: | gingival overgrowth |
| HIV: | human immunodeficiency virus |
| HMW: | high molecular weight |
| hs: | high sensitive |
| IL: | interleukin |
| KDa: | kilo Dalton |
| kg: | kilogram |
| LC-MS/MS: | liquid chromatography tandem mass spectrometry |
| LDS: | lithium dodecyl sulphate |
| LMW: | low molecular weight |
| m: | meter |
| mA: | milliamp |
| mg: | milligram |
| ml: | milliliter |

| | |
|---|---|
| mm: | millimeter |
| mM: | millimolar |
| MMP8: | matrix metalloproteinase-8 |
| MMP9: | matrix metalloproteinase-9 |
| MMW: | medium molecular weight |
| MPO: | myeloperoxidase |
| mRNA: | messenger ribonucleic acid |
| Nm: | nanometer |
| NSAID: | non-steroidal anti-inflammatory drug |
| OECD: | Organisation for Economic Co-operation and Development |
| OPG: | osteoprotegerin |
| OTM: | orthodontic tooth movement |
| PAR: | peer assessment rating |
| PAS: | Periodate Schiff Stain |
| PBS: | phosphate buffered saline |
| PDL: | periodontal ligament |
| pg: | pictogram |
| PG: | prostaglandin |
| PMN: | polymorphonuclear |
| PRAPg: | peroxisome proliferator-activated receptor gamma |
| qRT-PCR: | quantitative reverse transcription polymerase chain reaction |
| RANK: | receptor activator of nuclear factor kappa B |
| RANKL: | receptor activator of nuclear factor kappa B ligand |
| RCPCH: | Royal College of Paediatrics and Child Health |
| RP-HPLC: | Reversed-Phase High Performance liquid chromatography |
| SA-PE: | streptavidin-phycoerythrin conjugate |
| SD: | standard deviation |
| SDS-PAGE: | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SEM: | standard error of mean |
| S-HMW: | super high molecular weight |
| sIg: | secretory immunoglobulin |
| SWB: | Standard Western blot |
| TAD: | temporary anchorage device |

| | |
|---|---|
| TBST: | Tris Buffered Saline with Tween |
| TGF: | transforming growth factor |
| TIMP1: | tissue inhibitor of metalloproteinase |
| TNF: | tumor necrosis factor |
| UWMS: | unstimulated whole mouth saliva |
| VAS: | visual analog scale |
| WHO: | World Health Organisation |

# Acknowledgments

Firstly, I would like to acknowledge Allah for being with me through this endeavour and giving me the strength to accomplish my dream academically.

Big thanks for the Ministry of Higher Education and Scientific Research of Iraq for funding my PhD study as a part of educational scholarship programme.

I would like to express my extreme appreciation and respect to my supervisors, Prof. Martyn T. Cobourne and Dr. Guy H. Carpenter, for their enormous support and guidance along my PhD journey. I'm most grateful for the opportunity they have given me to enrol as a PhD student and for the encouragement when it was most needed.

Prof. Tim Newton, my postgraduate coordinator, thanks for being so caring since the first day we met and for your constant encouragement and support.

Prof. Gordon Proctor has been a great head of the mucosal and salivary biology division. He is not only always approachable, but also he never makes a person feeling silly for asking any question. Thanks for your time and support Prof. Gordon.

I would like to thank Dr. David Moyes, Dr. Elizabeth Witherden who were always available for major and small queries and gave me precious advice; and Dr. Spyridon Papageorgiou for statistical consultation.

Special to for Dr. S. AL-Hanoush, she was always happy to help and I will always be grateful for her support, encouragement, time and friendship.

My PhD would not be a good experience without all the supportive people I have had the pleasure to work with within the mucosal and salivary biology division and postgraduate orthodontic clinic. Also, I would like to sincerely thank all my fellow PhD students for their friendship and advice along the way, all the volunteers and patients who kindly participated in my study, and all the clinical staff, dental laboratory staff and students at Guy's Hospital, King's College London Dental Institute for their cooperation during my clinical work.

Lastly, I want to thank my family to whom I dedicate this thesis. Words cannot express the appreciation I have for everything my parents and sisters have done and scarified. They are always there when I need their support. Their support has meant everything to me and I really cannot do anything in my life without them.

# Chapter 1 Introduction

## 1.1 Obesity

Obesity is a specific disease state characterized by chronic subclinical inflammation mediated through excess adipose accumulation and the concomitant production of systemic pro-inflammatory factors (Deng et al., 2016). There has been a steady rise in obesity during the last few decades (Ng et al., 2014). This trend represents a major healthcare challenge over both the short and long-term because of the significant associations between obesity and multiple chronic diseases (Whitlock et al., 2009). It is widely believed to be a key risk factor for various conditions, including type 2 diabetes (Lean et al., 1998), cardiovascular disorders such as congestive heart failure, hypertension and coronary artery disease, and both obstructive sleep apnoea and cardiac arrhythmias (Kopelman, 2000, Willett et al., 1995) and overall increased risk of all-cause mortality (Aune et al., 2016); however, the causal mechanisms behind these relations remain uncertain. Although these risks are more widespread in adults, they are growing in obese adolescents, with sleep apnoea in particular causing increased concern (Neeley II and Gonzales, 2007). Obesity is also known to influence systemic bone metabolism through complex mechanical, hormonal, and inflammatory interactions (López-Gómez et al., 2016) with associations between obesity and reduced bone remodelling (Ivaska et al., 2016) and increased bone mineral density (Salamat et al., 2016). Although there is little data relating obesity to changes in alveolar bone composition within the healthy periodontium, longitudinal data has shown a significant association with increased rates of tooth eruption (Must et al., 2012).

Numerous studies have shown that obesity is not exclusive to the adult population in developed countries; a large proportion of children and adolescents are now showing significant levels of fat accumulation (Kopelman, 2000, Fadel et al., 2013). One of the greatest concerns with childhood obesity is that an obese child tends to develop into an obese adult who will exhibit all the associated co-morbidities (Pietrobelli et al., 2005).

### 1.1.1 Prevalence of obesity

In 2011, the Organisation for Economic Co-operation and Development (OECD) published data regarding both overweight and obese populations and did so for varying countries. Based on a series of up-to-date health surveys in the OECD, 50.3% of the adult population reported that they were overweight or obese. India (2.1%), Indonesia (2.4%) and China (2.9%) were revealed to be the least obese countries, and the United States (33.8%), Mexico (30.0%) and New Zealand (26.5%) the most obese. Being overweight and obese has a prevalence of over 50% among adults in no fewer than 19 of 34 OECD countries. In Australia and New Zealand alone, the prevalence of obesity has more than doubled throughout the last 20 years, while it has increased by half in the United Kingdom and the United States (The NHS Information Centre, 2012).

Since 1995, England has seen a significant rise in childhood obesity, where 11% of boys and 12% of girls were obese. Obesity levels increased steadily up until approximately 2004 and 2005, when they peaked at 19% among boys and girls. Levels have been slightly reduced in recent years, implying a flattening trend or gradual downward shift. In 2014, the percentage of obese children was not statistically significant in relation to the previous years. However, in the same year, obesity among boys (19%) reached the same peak level as in 2004 (Fat, 2014) (Figure 1.1).

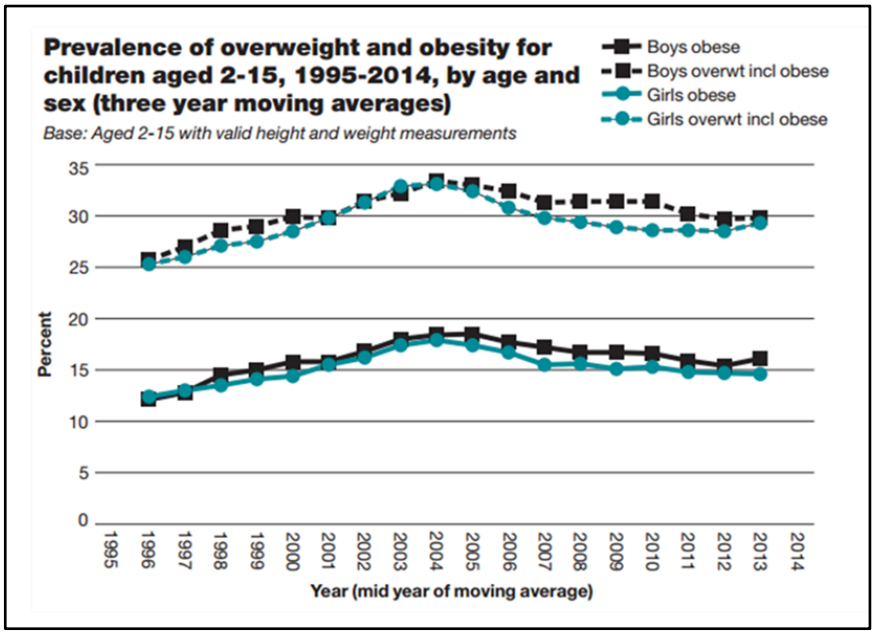



**Figure 1.1** Three-year moving averages from 1995 to 2014 for children aged 2-15 who were obese, and overweight including obese. Acknowledgments to (Fat, 2014).

Globally, it is assumed that 287 million school-age children can be classed as severely overweight (Von Bremen et al., 2012) and that this global tendency is on the increase. The International Obesity Task Force has stated that in 2004 approximately 14 million school children in the European Union were overweight, with 3 million of these being classed as obese (Lobstein et al., 2004). The same pattern is clear when looking at the United States data collected in the National Health and Nutrition Examination Survey. While only 4.6% of American adolescents were overweight from 1963 to 1965, this number had risen to 15.5% by 2000 (Ogden et al., 2002). It then continued to increase to 17.1% in 2004, before reaching 18.1% in 2008 (Ogden et al., 2010). Indeed, other authors report much higher values, with data collected from the United State or Brazil indicating that almost 30% of children and adolescents are overweight or obese (Flores et al., 2013).

### 1.1.2 Causes of obesity

A wide range of risk factors have been associated with obesity, including single gene mutations (Farooqi et al., 2003), gene-environment interactions (Qi et al., 2014). Environmental factors also play a significant role in causality, including a sedentary lifestyle, the intake of high-energy foods (Kopelman, 2000, Baur and O'Connor, 2004), sugary soft drinks (Ludwig et al., 2001) and a lack of physical activity (Robinson, 2001) among many other factors. Collectively, these may affect leptin sensitivity, which plays a role in the pathogenesis of obesity (Friedman, 2000). Indeed, these adverse lifestyle patterns are often established from early childhood (Reilly et al., 2005).

Although the mechanisms that underpin obesity remain unclear, it is understood that genetic and environmental factors, as well as socioeconomic and behavioural influences, lead to increased caloric intake and reduced physical activity. Furthermore, such factors prompt metabolic and endocrine abnormalities, both of which represent important causative elements. Other studies reported that obesity may be influenced by the pathology caused by oral bacteria that increase metabolic efficiency and appetite, and redirect the metabolism of energy through increasing levels of TNF-α or decreasing adiponectin levels (Goodson et al., 2009).

### 1.1.3 Assessment of obesity

The term "overweight" denotes an excess of body weight, whereas "obesity" indicates an excess of fat. Direct measures of body fat are not available clinically, meaning that obesity is assessed by anthropometrics that indirectly estimate body fat (Flodmark et al., 2004).

Assortments of methods have been described to assess obesity for both research and clinical purposes. First, the triceps skinfold uses a measurement obtained by grasping the skinfold at the triceps or subscapular area (Goran, 1998), it provides information about overweight children more effectively than it does for the presence of fat (Freedman and Sherry, 2009). Second, waist circumference measures obesity at the narrowest area, between the iliac crest and the rib cage, using a measuring tape (Roche et al., 1996), it correlates predominantly with cardiac disorders and an adverse lipid profile (Ramachandran et al., 2002). The third and most commonly used method in the assessment of obesity is Body Mass Index (BMI). This constitutes a numerical index that classifies the weight of an individual via the formula: BMI=weight in kilograms/squared height in meters (Kopelman, 2000, Ogden et al., 2006). Body weight is then classified using a range from underweight to obese, making the BMI a standard method for the evaluation of the body weight of an individual and its subsequent comparison with that of other individuals.

According to the World Health Organization (WHO), an adult person is considered underweight if their BMI is <18.5; healthy (normal weight) if the BMI is 18.5-24.9; overweight if the BMI is 25-29.9; obese if the BMI is 30-39.9; and morbidly obese if the BMI is ≥ 40. BMI should take into consideration age and gender for individuals of 2-20 years and express the results in a percentile. This means that for children, specific cut off points for the BMI are absent and instead a percentile is relied upon (Pietrobelli et al., 2005). The validity of the BMI has been investigated extensively and presents limitations in terms of overestimating body fat in people with unusually large muscle mass, as is frequently the case for trained athletes or body builders (Deurenberg et al., 1999).

### 1.1.4 Obesity and inflammation

Obesity has been shown to increase susceptibility to infection via the modulation of immune responses (Falagas and Kompoti, 2006). Diverse inflammatory mediators are released by adipocytes, provoking a systemic inflammatory state that may negatively affect the healing of wounds (Maury and Brichard, 2010, Ouchi et al., 2011).

Adipose connective tissue exhibits a predominance of adipose cells (adipocytes), which are found either in clusters or in isolation within connective tissue. Due to the majority of these cells being present in large aggregates, they constitute a major constituent of adipose tissue throughout the body and can be split into two categories: unilocular (common or yellow) and multilocular (brown). Unilocular adipose tissue comprises cells containing one large central droplet of yellow fat in their cytoplasm. Multilocular adipose tissue comprises cells that contain numerous lipid droplets and abundant brown mitochondria, which are chiefly involved in thermogenesis in the neonate (Unqueira and Carneiro, 2005).

Adipose tissue acts as an endocrine organ by secreting numerous adipokines that can cause a range of diseases due to dysregulated immune responses (Ouchi et al., 2003, Ouchi et al., 2011). Adipokines are soluble proteins that bind to specific receptors on target cells – usually cells that surround their producing cells – and induce the production of intracellular signals. This leads to changes in the cells as a result of alterations in their gene expression and regulation. Adipokines are effective even at low concentration and play an essential role in inflammatory disorders, such as periodontal disease (Ouchi et al., 2011). Similar to cytokines, they influence the secretion of other adipokines within networks. To take an example, IL-1β and TNF-α are able to act in unison to stimulate the production of IL-6 by human gingival fibroblasts (Ouchi et al., 2011). Also IL-8 and TNF-α can prompt the accumulation of neutrophils within the tissues of periodontitis (Liu et al., 2001).

The relationship between obesity and adipokines has been studied extensively. Leptin is secreted by adipocytes, regulating appetite and metabolism through hypothalamic mediators (Zhang et al., 1994). Moreover, inflammatory cytokines such as TNF and IL-1 are produced by adipocytes and lead to the pro-inflammatory state associated with obesity (Fantuzzi, 2005). Conversely, a significant reduction of IL-6 and TNF-α, coupled by an increase in IL-8 levels in the plasma, has been identified with weight loss (Bruun et al., 2003). A study of young subjects shows a positive correlation between TNF-α in GCF and a BMI $\geq$ 40, as well as with non-pathological periodontal pockets. This indicates that increases in TNF-α are due to a systemic effect, rather than being a result of the monocytes found in inflammatory periodontal conditions (Lundin et al., 2004). An alternative study evaluated the volume of adipocytes and the relation that this has with TNF-α, IL-6, adiponectin and high sensitivity C-reactive protein (hs-CRP) in obese and lean patients both with and without type 2 diabetes. The study revealed that adipocyte volume is

positively correlated with TNF-α, hs-CRP and IL-6 levels, yet negatively correlated with adiponectin levels. Furthermore, it is important to emphasize that this correlation was not only related to type 2 diabetes but also to obesity when analyzed alone (Bahceci et al., 2007).

### 1.1.5 Obesity and bone metabolism

Obesity and bone metabolism are also interrelated, with obesity affecting bone metabolism through a range of mechanisms. Since adipocytes and osteoblasts are derived from a common mesenchymal stem cell (Gregoire et al., 1998), with the agents that inhibit adipogenesis stimulating osteoblast differentiation (Gimble et al., 1996, David et al., 2007b, Sen et al., 2008) and those that inhibit osteoblastogenesis increasing adipogenesis (Beresford et al., 1992); obesity may decrease osteoblastogenesis, while increasing adipogenesis. Mechanical loading promotes osteoblast differentiation and inhibits adipogenesis through the down-regulation of the peroxisome proliferator-activated receptor gamma (PPARg) or the stimulation of a durable beta-catemin signal (David et al., 2007b, Sen et al., 2008). The activation of PPARg by thiazolidinediones decreases osteoblast differentiation, bone mineral density and trabecular bone mass, while increasing adipocyte differentiation and bone marrow adipose tissue volume (Gimble et al., 1996, Tornvig et al., 2001, Lazarenko et al., 2006).

Furthermore, bone resorption may be increased by obesity by way of up-regulation of pro-inflammatory cytokines, such as IL-6 and TNF-α. Both of these have been shown to be capable of stimulating osteoclast activity through regulation of the RANKL/RANK/OPG pathway (Khosla, 2001, Pfeilschifter et al., 2002) (Figure 1.2).

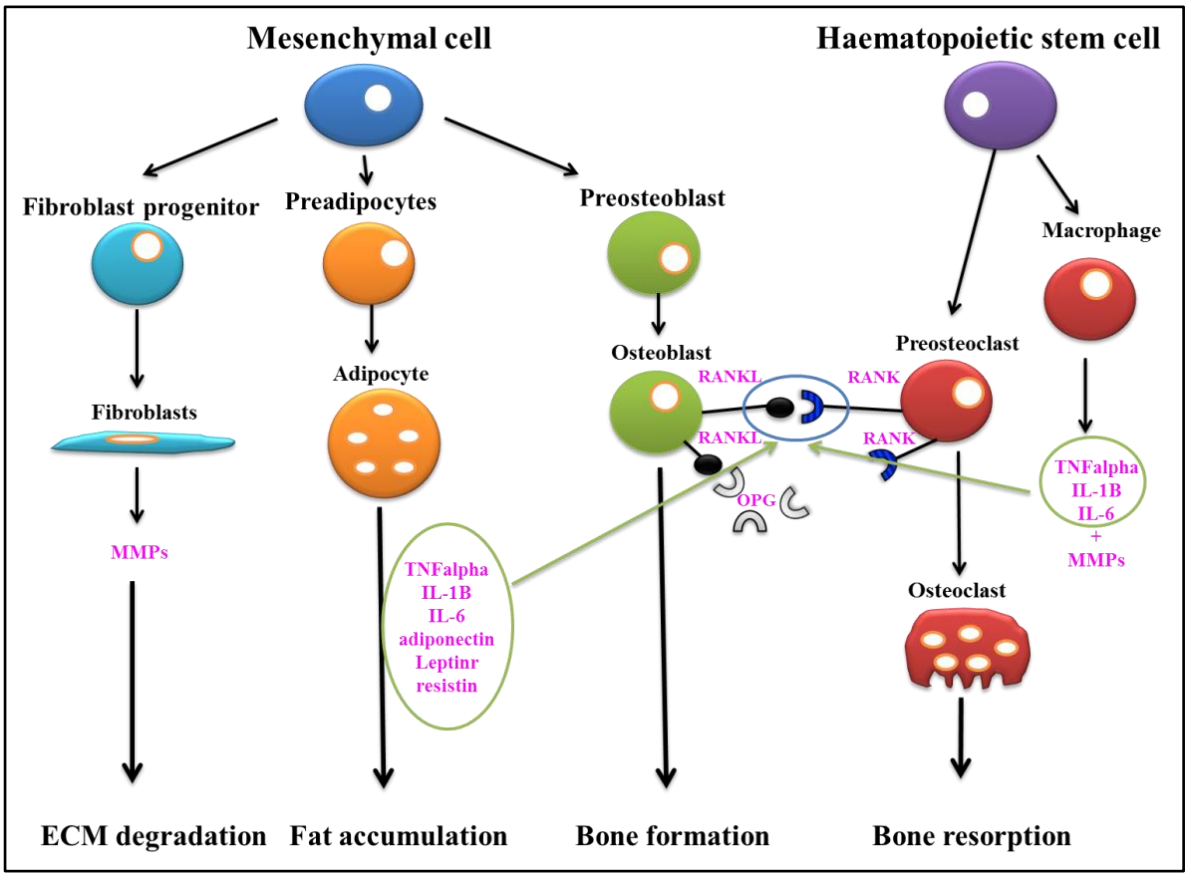



**Figure 1.2** Bone resorption upregulated by adipocytes. Adipocytes secret TNF-α, IL-1β, IL-6, adiponectin, resistin, and leptin are capable of modulating osteoclastogenesis through RANKL/RANK/OPG pathway (grey-green arrows). Osteoblasts, fibroblasts and adipocytes are derived from mesenchymal stem cell. Osteoclasts are differentiated from monocyte/macrophage precursors of hematopoietic stem cells origin. IL, interleukin; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor kappa B; RANKL, receptor activator of nuclear factor kappa B ligand; TNF-α, tumor necrosis factor alpha; MMP, matrix metalloproteinase; ECM, extracellular matrix. Adapted from (Cao, 2011).

Obesity may have an effect on bone metabolism, either directly or indirectly, due to adipocyte-derived adipokines, such as leptin and adiponectin. Both an increase in serum leptin (Canavan et al., 2005, Van Dielen et al., 2001) and a decrease in serum adiponectin (Ouchi et al., 1999) have been linked to obesity that may stimulate the transportation of macrophages to adipose tissue (Sierra-Honigmann et al., 1998), thus promoting their adhesion to endothelial cells (Maeda et al., 2002) (Figure 1.3).

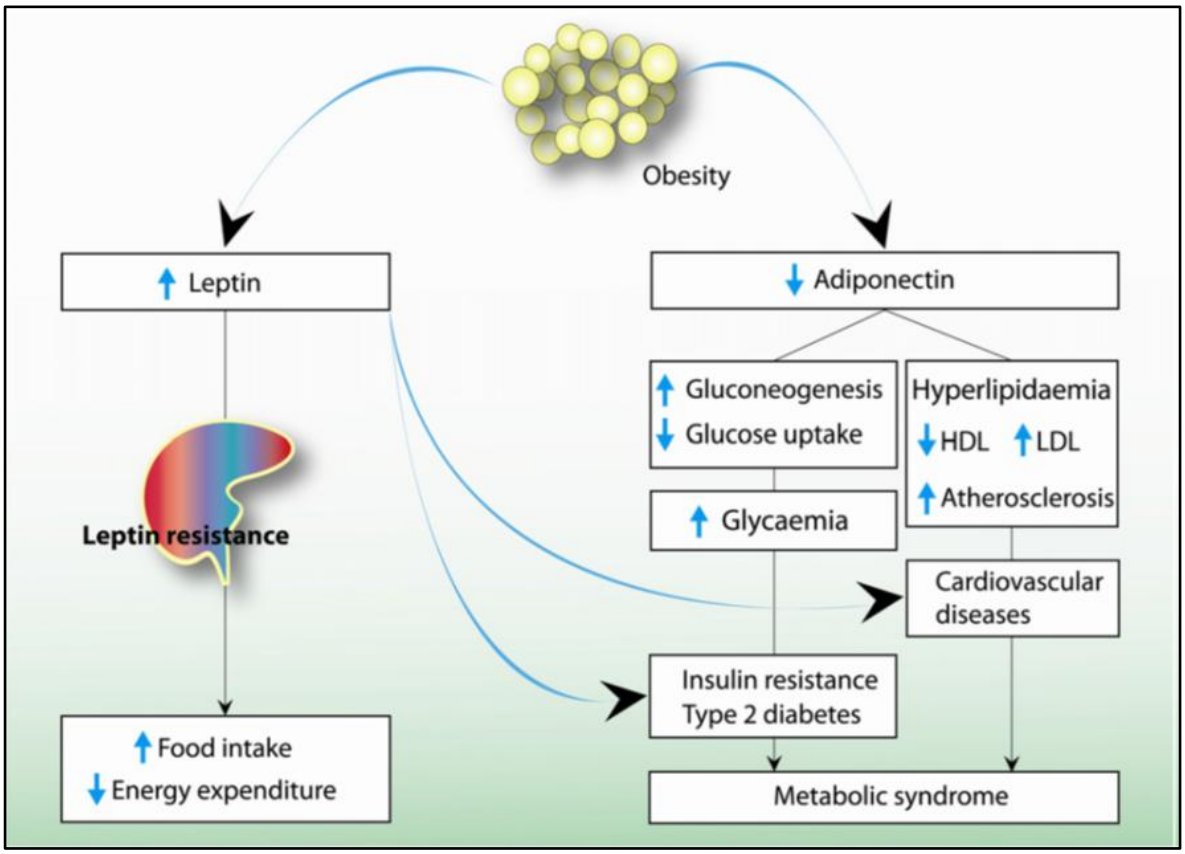



**Figure 1.3** The implication of leptin and adiponectin in the pathogenesis of the metabolic syndrome in obesity. Acknowledgments to (Ricci and Bevilacqua, 2012).

Finally, it has been shown that a high-fat diet, which is often a cause of obesity, interferes with the absorption of calcium in the intestines. Free fatty acids have the potential to form insoluble calcium soaps that cannot be absorbed, thus encouraging low calcium absorption (Carnielli et al., 1996, Nelson et al., 1998).

In general terms, body weight is positively correlated with bone mass (Felson et al., 1993, Reid, 2002) and low body weight presents a risk factor in terms of depleted bone mass and increased bone loss in humans (Ravn et al., 1999). However, decreased bone mass as a result of obesity may be due to increased marrow adipogenesis at the expense of osteoblastogenesis, and/or increased osteoclastogenesis due to an increase in the production of pro-inflammatory cytokines. Excessive leptin secretion, or reduced adiponectin production, and/or reduced calcium absorption associated with a high fat intake also present possible triggers for obesity-related decreased bone mass.

### 1.1.6 Obesity and periodontal diseases

Adipose tissue can influence the intensity and resolution of inflammatory responses in multiple tissues (Issa and Griffin, 2012, Pierpont et al., 2014). Indeed, there is evidence of an increased risk of chronic periodontitis (Keller et al., 2015, Suvan et al., 2011) and variation in inflammatory and metabolic markers in obese subjects affected by periodontal disease when compared to normal weight (Papageorgiou et al., 2015a). The systemic induction of inflammatory markers may provide a link between obesity and periodontitis, (Pradeep et al., 2012).

Studies into the association between obesity and periodontitis as a chronic problem in health are frequently cited in the literature (Al-Zahrani et al., 2003, Sarlati et al., 2008, Ylöstalo et al., 2008). First observed in 1977 in rats, bone resorption and periodontal inflammation have been shown to be more severe in obese populations (Perlstein and Bissada, 1977). These early findings were supported by further studies (Khader et al., 2009, Ritchie, 2007) that show obesity is a predictive factor for periodontal disease regardless of age, gender, race, ethnicity or the effects of smoking. It is possible that this can be explained by the hyper-inflammatory nature of obesity, which may lead to a resistance to insulin and eventually prompts both a greater susceptibility to diabetes and an increased risk of periodontitis (Lakkis et al., 2012).

Longitudinal studies have found a direct association between obesity and overweight individuals at the baseline with subsequent advances in periodontitis (Jimenez et al., 2012, Gorman et al., 2012). The results of one particular review into the association between periodontal disease and overweight and obese individuals suggests that being overweight, obesity, weight gain and increased waist circumference may all constitute risk factors for the development or worsening of periodontal measures, such as probing depth, alveolar bone loss, attachment loss and plaque index (Keller et al., 2015).

The risk of periodontal disease and obesity is increased by diabetes, smoking, osteoporosis, alcohol consumption, psychological stress and age (Genco, 1996, Genco and Borgnakke, 2013). It has also been reported that people with a high BMI suffer from periodontitis, with significant clinical attachment loss in adults (Suvan et al., 2011, Chaffee and Weston, 2010) and similarly in children (Ekuni et al., 2008, Modéer et al., 2011). These findings have been linked to the exacerbated pro-inflammatory changes that take place with periodontal disease.

The underlying biological mechanisms behind any association between obesity and periodontitis are not well known. Oxidative stress is thought to link periodontitis and a range of metabolic disorders, including high blood pressure and impaired glucose regulation, which is predominantly related to a chronic pro-inflammatory state (Eckel et al., 2005). Additionally, it has been hypothesized that an association between obesity and periodontal disease may result in a host defence alteration, an impairment of glucose tolerance, an increase in the psychological stress response and pro-inflammatory response secretion (Range et al., 2013).

A more recent study estimates the local and systemic concentrations of resistin, adiponectin, leptin, TNF-α, IL-6 in serum and GCF in obese and normal weight people, both with and without chronic periodontitis. This specific study demonstrated that levels of resistin increase with periodontitis whereas adiponectin levels decrease. In addition, higher levels of TNF-α were noted in obese patients with the presence or absence of periodontitis (Zimmermann et al., 2013). These results are further confirmed by research that establishes an association between TNF-α and BMI (Lundin et al., 2004).

Obese and normal weight males were compared in further investigations into the field, with this particular study concluding that leptin levels were affected by both periodontitis and obesity, with marked differences seen in IL-6 levels among the tested groups (Khosravi et al., 2009). These outcomes were confirmed by other studies which stated that the secretion of TNF-α and IL-6 is a result of the pro-inflammatory effect of resistin and consequent impairment effect of adiponectin (Bokarewa et al., 2005). Since TNF-α plays a role in both bone matrix degradation and bone resorption (Kurtis et al., 2005), periodontal breakdown can indeed be expected, even where clinical signs remain absent, in obese patients both with or without periodontitis (Rossomando et al., 1990).

In addition, periodontitis has been found to be 1.8 and 2.3 times more frequent in obese and overweight/obese individuals than individuals of normal weight, respectively. The related mechanism is enhanced by additional inflammatory responses and an increase in the quality and quantity of dental plaque in obese persons (Suvan et al., 2014). Consequently, host response to the antigens derived from bacterial plaque may be altered by obesity, prompting instabilities in the inflammatory response throughout the duration of periodontal disease (Słotwińska and Słotwiński, 2015). As an inflammatory disease then, periodontitis may be influenced by local and systemic factors, including diabetes, immune disorders, smoking and obesity.

### 1.1.7 Obesity and orthodontics

In light of the results outlined above and further studies detailed in the literature below, obesity may significantly affect orthodontic therapy via its effect on bone metabolism, growth and development, which consequently affects puberty and potentially the movement of teeth during orthodontic therapy in obese persons (Neeley II and Gonzales, 2007). Furthermore, obesity can have a significant and negative psychological effect on children. Obese children tend to be socially isolated, a factor that may explain their tendency to fail to comply with orthodontic therapy (Must and Strauss, 1999). Perhaps more drastically, the facial aesthetics of obese individuals differ from those of people with normal weight, which is a factor that should be taken into consideration when planning orthodontic treatment. For instance, cephalometric and facial analyses should be altered during the examinations of obese or overweight patients. Such patients tend to exhibit larger mandibles and shorter distance in the upper face that have the potential to alter treatments. Obese patients also tend to have flatter or more concave profiles as a result of increased mandibular length and increased tissue thickness. The average or normal values for this group should be viewed in light of the knowledge that they were originally acquired through the measuring of persons of normal weight and that therefore they might not apply to obese populations. In light of this information, such patients should be treated individually with careful attention being paid to their psychosocial status (Neeley II and Gonzales, 2007).

An alternative research paper studied the impact of BMI on oral health and patient cooperation during orthodontic treatment. It found that an increased BMI seems to be a risk factor for reduced cooperation, longer treatment duration, and an increased number of oral health-related problems during multiple bracket treatment. This indeed indicates that such patients need special attention during orthodontic therapy (von Bremen et al., 2015). Another study in the field has been undertaken to quantify the association between BMI and the wear time of removable orthodontic appliances, as well as assess alterations in BMI during orthodontic treatment. This particular study failed to find statistically significant or clinically relevant differences for usage or adherence in normal-weight and overweight / obese patients. This suggests that orthodontic treatment in young patients with removable devices does not require BMI-dependent changes to be taken into account in the treatment strategy. However, the study does state that the use of removable appliances during meal times raises the likelihood of a reduction in food intake, meaning that the orthodontist may

play an active role in weight reduction (Schott and Ludwig, 2014). Another study related to this theme has been undertaken into the relationship between BMI percentile and skeletal maturation, using the cervical vertebral method, and dental maturity, using the Demirjian assessment method, in adolescent orthodontic patients. This particular work found the cervical vertebral stage and dental age to be more advanced in subjects with increased BMI percentiles. As a result, the recommendation is that orthodontists consider weight when evaluating growing children and adolescents (Mack et al., 2013).

Periodontal-orthodontic therapy is usually aimed at the correction of a periodontal condition, aesthetic and functional improvement. Although some believe that orthodontic treatment may worsen the periodontal status of a patient (Zetu et al., 2010), a selection of other researchers assert that osteogenetic variations between periodontitis and non-periodontitis patients may result in differences in orthodontic treatment (Dersot, 2012).

## 1.2 Biomarkers

A biomarker is a substance that is objectively measured and evaluated as an indicator of physiologic, pathogenic conditions or outcome to a therapeutic treatment (Taba et al., 2005).

### 1.2.1 Cytokines

Cytokines are low-molecular weight proteins released by specific cells that prove to be effective in very low concentrations. Their actions may play out locally or systemically, either on the very cell that secretes the cytokine (autocrine) or on a nearby cell (paracrine) (Okada and Murakami, 1998). Cytokines are produced through a cascade process in which, one cytokine stimulates its target cells to make additional cytokines. Cytokines are also able to act in a synergistic (two or more cytokines acting together) or antagonistic manner (cytokine causes opposing activities) (Fuhlbrigge et al., 1997). Multiple cytokines operate as a network in a pleiotropic (that is, the ability of one cytokine to act on different cell types) and redundant (refers to the property of multiple cytokines having the same functional effectors) (Fuhlbrigge et al., 1997, Prlic and Bevan, 2006). Cytokines are known to have a short half-life that ranges from a few hours to a few days, with a low concentration of plasma ensuring that it acts for a limited period of time only and thus across short distances (Kindt et al., 2007).

Immune system cells produce several types of cytokine, including lymphokines, monokines and interleukin cytokines, which are made by lymphocytes, monocytes and a certain form of leukocyte, respectively. The chemokines cytokine is responsible for a range of chemotactic activities within the human body (David et al., 2007a).

Cytokines act directly or indirectly in the enabling of bone and periodontal ligament (PDL) cell differentiation, activation and apoptosis and are involved in the bone remodelling and inflammatory processes during OTM (Krishnan and Davidovitch, 2006a, Meikle, 2006). Various researchers have demonstrated elevated levels of cytokines in tooth movement (Alhashimi et al., 2001); more specifically, cytokines are most commonly present in the PDL during the early stages of OTM (12-24 hours). Extended investigations into the mechanisms through which they have identified both their effector (pro-inflammatory) and suppressive (anti-inflammatory) functions during OTM (Lynch et al., 1988). Due to sequential reactions and released substances, several of these biologically active molecules have been proposed as suitable biomarkers that may aid in developing a better understanding of the biological processes involved in OTM, improving treatment and reducing adverse side effects (Zainal Ariffin et al., 2011).

The trend of obesity has sharply risen and became an increasing concern. This accompanied by an interest in the immunomodulatory cytokines as potential mediators and/or targets for prevention and treatment of obesity and metabolic syndromes (Pasquin et al., 2016), especially there is evidence of markedly increase of TNF-α, IL-6, and IL-8 release by adipose tissue of obese subjects (Fain, 2006).

### 1.2.2 Adiponectin

Adiponectin is a protein released by adipocytes that exhibits various functions in the targeting of different types of cells. To be more specific, it has been shown to potentially influence insulin resistance, inflammation and cardiovascular systems (Funahashi et al., 1999), and may even harbour various beneficial effects for obesity and obesity-related diseases (Turer and Scherer, 2012). AdipoR1 and ApidoR2 are the names of the two adiponectin receptors that have been discovered and they are expressed in skeletal muscles and the liver, respectively (Yamauchi et al., 2003). Both of these receptors exert anti-inflammatory effects and are also expressed in the fibroblasts of gingiva and PDL (Iwayama et al., 2012). A third receptor, known as T-cadherin and mainly associated with

middle- and high-molecular weight adiponectin, is expressed on vascular endothelial cells and smooth muscle (Hug et al., 2004). When AdipoR1 and R2 are activated, an increase in the oxidation of fatty acid in skeletal and hepatic muscle results, as well as increased lactate production by skeletal muscle, reduced hepatic gluconeogenesis, increased glucose uptake and the suppression of inflammatory and oxidative stress (Yamauchi et al., 2002, Yoon et al., 2006). Moreover, when T-cadherin is activated, the vascular endothelial cells are protected against apoptosis induced by oxidative stress (Joshi et al., 2005).

Adiponectin produced and secreted mainly by adipocytes (Scherer et al., 1995). It has N-terminal collagen-like region and a C-terminal complement factor C1q-like globular domain (Shapiro and Scherer, 1998, Kishore et al., 2004, Wong et al., 2004). Thus, it exists as a full-length protein and/or globular adiponectin. This cleavage is mediated by a leukocyte elastase, secreted by activated monocytes and/or neutrophils (Tilg and Moschen, 2006). Serum adiponectin is produced as a monomer of 28 kDa. By way of a number of post-translational modifications, self-assembly then occurs as oligomers of differing molecular weights: high molecular weight (HMW), medium molecular weight (MMW), and low molecular weight (LMW) (Kadowaki and Yamauchi, 2005, Daniele et al., 2008a, Liu and Liu, 2014) (Figure 1.4). The oligomeric state of adiponectin is particularly important due to the fact that it has a significant influence on biological activity (Schapher et al., 2009). More specifically, various in vitro studies reveal HMW to be the most biologically active of oligomers. These are, in turn, associated with metabolic disorders and thus provide us with an indispensible diagnostic marker (Hirose et al., 2010).

Evidence suggests that salivary gland epithelial cells are responsible for producing adiponectin and may indeed be involved in local immune response regulation. Salivary adiponectin measurement is easy and non-invasive, and would be useful if it were to be included in a range of clinical studies. The occurrence of adiponectin and very high molecular weight oligomers in saliva has been mentioned in at least one study (Kadowaki and Yamauchi, 2005). Alternative studies have measured salivary adiponectin levels in saliva samples collected using two different methods for each participant, namely the test tube and the Salivette systems (cotton wads). The results were then correlated with serum adiponectin, the researchers found a significant correlation between adiponectin levels in plasma and test-tube saliva, while a correlation between adiponectin levels in plasma and the Salivette-sampled saliva remained absent (Toda and Morimoto, 2008). A further research paper and investigation found an increase in measurable levels of adiponectin in

saliva upon the dilution of the sample. This specific paper goes on to explain that due to inhibitor(s) of adiponectin/anti-adiponectin binding in the saliva, after dilution the inhibitory effect becomes clear (Akuailou et al., 2013). Further research has observed adiponectin in both human serum and saliva via a process of Western blotting under non-reducing conditions. This revealed a detection rate of serum adiponectin of 150 kDa (hexamer) and 250 kDa (high molecular weight multimer), with only one band having been detected in saliva that sits above the higher molecular band found in serum and referred to as the super higher molecular band (Lin et al., 2014). A number of studies have found very low adiponectin levels in human saliva when studied in relation to plasma (Toda et al., 2007, Toda and Morimoto, 2008, Akuailou et al., 2013).

With regard to BMI, a series of studies have shown that serum adiponectin levels and the amount of oligomers are inversely correlated with BMI, as well as being very closely related to obesity and related diseases (Daniele et al., 2008b, De Rosa et al., 2013). A further study has analyzed total adiponectin expression and its oligomeric profile in samples of saliva for both obese subjects and controls. The same study also compared adiponectin oligomerization between samples of serum and saliva. The paper's analysis of the different adiponectin oligomers reveals a slightly higher expression of total, HMW and LMW salivary adiponectin in obese patients compared to the controls. Additionally, HMW oligomers found in saliva have a higher molecular weight than those found in serum, which confirms that more complex oligomers can be found in saliva, known as super HMW (S-HMW) (Nigro et al., 2015).

Obesity may trigger a decrease in both plasma adiponectin and its receptors (AdipoR1 and AdipoR2), thus leading to adiponectin resistance and subsequent insulin resistance (Kadowaki and Yamauchi, 2005). Alternative studies reveal that patients with diabetes type 2, obesity and coronary artery disease may exhibit hypo-adiponectinemia (Ouchi et al., 1999, Joseph et al., 2002).

In periodontitis, adiponectin may not work effectively, since a reduced number of adiponectin receptors may in turn worsen periodontal disease (Yamaguchi et al., 2010). This is confirmed by other studies that have found a reduced number of adiponectin receptors in patients with severe periodontitis when compared to their healthy counterparts (Saito et al., 2008, Yamaguchi et al., 2010). Moreover, since adiponectin and its receptors are expressed in osteoblast cells, it is believed to be linked to both anti-inflammatory functions and bone metabolism (Berner et al., 2004, Oshima et al., 2005). It may affect the

function of PDL cells, which may be differentiated between as osteoblasts and cementoblasts (Seo et al., 2004). Additionally, one particular study has revealed that adiponectin has anti-inflammatory properties for human and mouse gingival fibroblasts, while also improving the osteoblastogensis of PDL cells in humans. It also helps to maintain the homeostasis of periodontal health, improve periodontal lesions, and promote wound healing and tissue regeneration (Iwayama et al., 2012). A selection of other studies shows that the serum concentration of adiponectin is reduced in patients with periodontitis in comparison to healthy individuals (Saito et al., 2008, Furugen et al., 2008). These levels increase following periodontal treatment (Furugen et al., 2008).

Finally, research suggests that adiponectin may suppress TNF-α and stimulate inflammatory responses in macrophage and endothetial cells (Ouchi et al., 2011). It also has the potential to enhance the migration of endothetial cells and stimulate their differentiation into capillary-like structures (Ouchi et al., 2004).

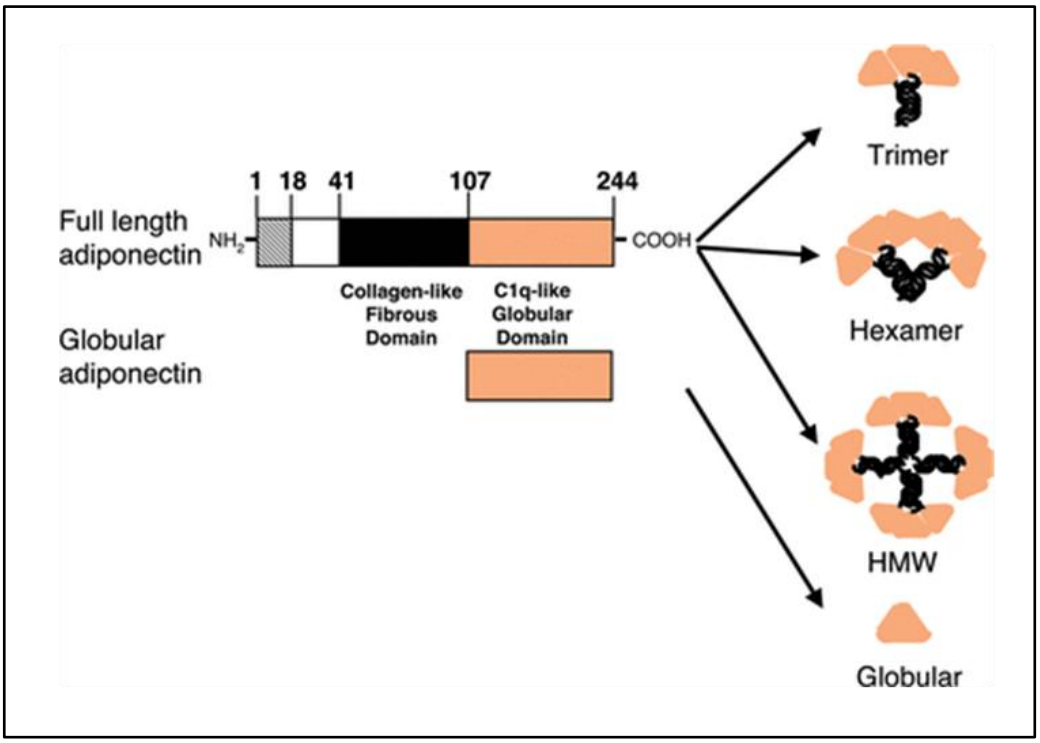



**Figure 1.4** Structure of adiponectin**.** Full-length adiponectin is composed of 244 amino acids, including a collagen-like fibrous domain at the N-terminus and a C1q-like globular domain at the C-terminus. In plasma, full-length adiponectin combines via collagen domain and forms multimer complexes, such as trimers and hexamers, and a high-molecular-mass (HMW) form. A smaller form of adiponectin that consists of globular domain also exists in plasma in very small amounts. Acknowledgments to (Okamoto et al., 2006).

### 1.2.3 Leptin

Leptin is an adipose-derived hormone secreted in proportion to the size and number of adipocytes present. As a result, plasma leptin levels are higher for obese individuals and decreased after weight loss is achieved (Considine et al., 1996). While leptin is mainly synthesized by adipose tissue it is also produced at low levels in other tissues. Leptin has a range of effects, including appetite inhibition, energy expenditure stimulation, and the modulation of lipid and bone metabolisms, coagulation, hematopoiesis, pancreatic beta-cells function and insulin sensitivity (Krysiak et al., 2012, Carbone et al., 2012) (Figure 1.5). Moreover, leptin is known to regulate the immune system and inflammatory responses via chiefly pro-inflammatory actions (Krysiak et al., 2012, Carbone et al., 2012, Procaccini et al., 2012). Leptin mediates its effects by binding to the full-length leptin receptor and, in addition to the brain; this receptor is also expressed in a wide range of peripheral tissues. Upon binding, various intracellular pathways and transcription factors are triggered (Carbone et al., 2012, Zhou and Rui, 2013).

A polypeptide hormone, leptin is also categorized as a cytokine (Zhang et al., 1994), and its receptor shares structural and functional likenesses with various long-chain helical cytokines: IL-6, IL-11, IL-12, leukaemia inhibitory factor, granulocyte-colony–stimulating factor, and oncostatin M (Ahima and Flier, 2000). Leptin is said to orchestrate the host reaction to both inflammatory and infectious stimuli, since it stimulates the immune system by boosting cytokine production and phagocytosis by macrophages (Fantuzzi and Faggioni, 2000). Accordingly, an overall upsurge in leptin during inflammation and infection suggests that leptin is a component of the mechanisms of both immune response and host defense.

Earlier studies signal a relationship between periodontal disease and leptin levels due to successful demonstrations of the presence of leptin in healthy and slightly inflamed gingivae (Johnson and Serio, 2001). Numerous investigations show that levels of GCF leptin activity may shape the development of periodontal disease, with its level decreasing progressively in GCF as periodontal disease progresses (Karthikeyan and Pradeep, 2007b).

It has also been suggested that leptin contributes to bone formation due to its direct effect on osteoblast proliferation and differentiation, as well as its prolonging of the life span of human primary osteoblasts by inhibiting apoptosis (Bozkurt et al., 2006). Leptin is also involved in antiosteogenic effects since it acts centrally on the hypothalamus

(Włodarski and Włodarski, 2008). Overall, at high local concentrations leptin protects the host from inflammation and infection, while also maintaining bone levels.

A significant number of studies have investigated leptin in relation to orthodontic treatment. One such study tested whether leptin is detectable in GCF found around moving teeth. In order to determine whether any changes occur during OTM, a split mouth design study was undertaken during the distalisation of the canine at the baseline, after 1, 24 and 168 hours. Here, leptin concentrations of the test teeth were found to decrease in a time-dependent manner when compared to the baseline measurement, with the decrease becoming significant at 168 hours (Dilsiz et al., 2010).

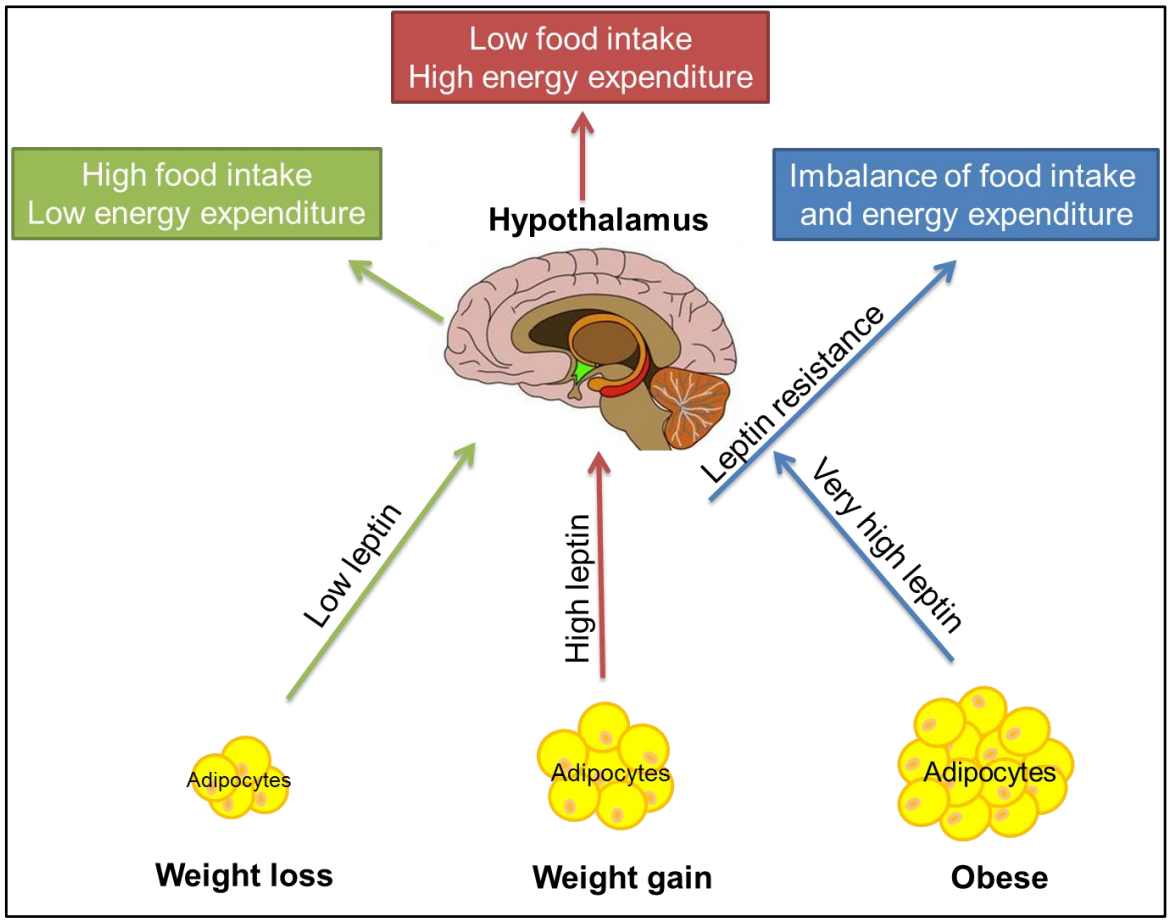



**Figure 1.5** The biology of leptin with different body weight. A loss of body fat causes a decrease in leptin, which leads to a state of positive energy balance where in food intake exceeds energy expenditure. An increase in body fat enhances the levels of leptin and a state of negative energy balance, with energy expenditure exceeding food intake. Obesity could result from insufficient or absence of sensitivity to leptin at its site of action that would increase circulating leptin.

### 1.2.4 Resistin

The biomarker resistin is a polypeptide derived from adipocytes and is named after its apparent ability to resist insulin. Rich in cysteine, this molecule belongs to the set of adipokines (which also includes leptin, adiponutrin and adiponectin) whose protein was first found in mouse adipose tissue. Also known as an adipocyte-specific secretory factor, only a very small amount of resistin is expressed in adipocytes in humans, and this chiefly takes place in the neutrophils, macrophages, and monocytes (Patel et al., 2003). Human resistin is also known to operate as a pro-inflammatory molecule, since it induces the synthesis and secretion of the following pro-inflammatory cytokines: TNF-α, IL-6, IL-12 and monocyte chemo-attractant protein (MCP)-1 (Silswal et al., 2005).

The link between periodontitis and resistin has been researched to a considerable extent in the field (Furugen et al., 2008). Resistin has also been studied in GCF in terms of its function as an inflammatory mediator in both the induction and resolution of human experimental gingivitis (Offenbacher et al., 2010). Rheumatoid arthritis, (Bokarewa et al., 2005), inflammatory bowel disease (Kaser and Tilg, 2008) and asthma (LaRochelle et al., 2007) (along with other chronic inflammatory conditions), also show a direct correlation with resistin concentration, and various studies indicate a positive correlation between resistin and pro-inflammatory factors in adults with patho-physiological conditions; these include, type 2 diabetes mellitus, atherosclerosis, inflammation of the respiratory tracts and renal disease (Brownlee, 1992, D'Aiuto et al., 2004).

One particular study detected the levels of resistin in the saliva and serum of diabetic type 2 and non-diabetic subjects using ELISA. Their results indicate the presence of resistin in the saliva of both groups, with a significantly higher level being registered for that of the diabetic patients. For type 2 diabetes mellitus, both salivary and serum resistin correlate positively with BMI and insulin resistance (Yin et al., 2012). A clinical study conducted to estimate the levels of resistin in GCF in health, chronic periodontitis and type 2 diabetes mellitus individuals suggests that resistin is found in GCF in all groups with a significant increase in periodontitis among diabetes mellitus groups (Gokhale et al., 2014). Another study compared resistin levels in GCF and serum in obese and non-obese subjects, both with and without periodontitis, found that the highest levels of resistin were observed in the obese group with periodontitis in both GCF and serum (Patel and Raju, 2014). Similarly, a more recent study measured the levels of resistin in obese and non-obese

subjects with and without periodontal disease, reported that resistin were elevated in GCF with obesity and periodontal diseases, therefore they recommended to consider resisitin as inflammatory marker as it was associated with both obesity and periodontitis (Suresh et al., 2016).

### 1.2.5 C-reactive protein

C-Reactive Protein (CRP) is present in the serum of individuals in the presence of illness and was first purified in 1941 (Burtis et al., 2012). It is produced in the liver as an inflammatory reactant during the acute inflammation phase. In biological terms, it exhibits pro-inflammatory effects and serves to intensify both the activation and infiltration of macrophages around necrotic tissue (Takahashi et al., 2010).

When acute inflammation and tissue destruction occurs, concentration levels for CRP rise within 6 to 24 hours up to levels of 1000 fold (Salonen et al., 1984). In the presence of chronic inflammation its presence is also known to increase, with its levels rapidly falling with the cessation of the stimulus (Håheim et al., 2009). The effects of cytokines such as IL-1, IL-6 and TNF-α are responsible for this elevation and are produced at the sites of inflammation before being carried to the liver via systemic circulation; this ultimately results in increased secretion of acute inflammatory reactants such as CRP (Ide et al., 2004, Gabay, 2006, Nibali et al., 2007).

CRP acts as a diagnostic marker for acute inflammation, tissue damage and low-grade systemic inflammation, with its values being used to supervise the progression of disease and the outcome of any treatment (Ridker and Silvertown, 2008, Paraskevas et al., 2008). Furthermore, CRP could be adopted as a predictive biomarker for both cardiovascular diseases and systemic inflammation (Danesh et al., 1998), which leads to the hypothesis that elevated levels in periodontitis could be related, at least in part, to a link between periodontitis and cardiovascular disorders (Paraskevas et al., 2008). More specifically, periodontal bacteria may be released into the blood stream through mastication, tooth brushing and dental treatments. These would then lead to a risk of bacteraemia, which is connected to the severity of gingival inflammation (Silver et al., 1977). Additionally, the cells present in the connective tissue that lies beneath periodontal pockets may discharge inflammatory biomarkers such as CRP, and even plasminogen activator 1 and fibrinogen (Blake and Ridker, 2002), leading as a result, to problems of a

systemic nature (Beck et al., 1996). The risk of cardiovascular disorder is considered low when the CRP serum level is less than 1 mg/l, intermediate at 1-3 mg/l, and high at above 3 mg/l (Ridker, 2003).

A positive link between CRP and oral disease has been identified in studies investigating the serum of systemically healthy persons experiencing oral inflammation (Boucher et al., 1967). Later research also notes that CRP levels in the entire saliva (Pederson et al., 1995) and GCF (Fitzsimmons et al., 2009, Fitzsimmons et al., 2010) of healthy subjects is correlated to a great extent with the severity of their periodontal parameters, namely plaque index, pocket depth and gingival index. However, it must be noted that GCF is a transudate of serum and thus contains both locally produced components and serum. As a result, the CRP level increase in GCF for periodontitis could be the result, in part, of local production within the gingival sulcus or production related to systemic origin (Black et al., 2004). One particular study dedicated to a specific comparison of the CRP in GCF and gingivae of subjects both with and without periodontitis shows that CRP in GCF and periodontal tissue is not of local origin. Instead, it is of a systemic origin that could result from periodontitis or a number of alternative systemic diseases. CRP production in tissue promoted from periodontium may provide an explanation for this, since it may occur as the result of locally produced biomarkers such as IL-1β (Marcaccini et al., 2010). Periodontitis can therefore be understood as a chronic disease characterized by bidirectional influences. Here, inflammation may occur due to a local increase in IL-1β production, which in turn may lead to a more systemic inflammatory response in remote organs such as the liver, to cite an example. The overall result of this would be an increase in CRP level in the serum. On the whole then, in a complex disease such as periodontitis both local and systemic factors may act both synergistically and independently (Fitzsimmons et al., 2010).

Since it induces both MMP1 and MMP9 in human endothelial cells, it may also have an important role in the connective tissue destruction in periodontitis (Caliskan et al., 2007). Additionally, the bacteria associated with periodontal disease that invade gingival tissues may interact in a direct manner with human arterial endothelial cells, which would lead to a higher local CRP level (Rautemaa et al., 2004, Jandik et al., 2008). However, this does not constitute the main source for a systemic elevation since here the levels are much lower than those seen in the blood. A clear and undisputed mechanism for the increase of CRP in patients with periodontitis has not been found. However, a variety of studies note

that IL-6 synthesis in the liver increases when sever periodontitis is present, which leads to the production of CRP (Slade et al., 2000, Noack et al., 2001). In such a case, the IL-6 source in cases of severe periodontitis could be either Kupfer cells in the liver and/or adipocytes (Yamaguchi et al., 2005).

In relation to this, one particular study was conducted in order to determine if the CRP detected in GCF is the product of local fabrication within the gingival tissue. The method used involved the collection of GCF and gingivae from non-periodontitis and periodontitis sites. The findings showed that CRP in the GCF appears to be of systemic origin, and therefore may be a sign of systemic inflammation due to periodontal infection or the presence of inflammatory disease elsewhere (Megson et al., 2010). A further study related to this field evaluated the effects of orthodontic treatment on systemic levels of CRP. To do so, the researchers compared CRP levels before treatment and then at 3 more time points, each set 2 months apart, during treatment using ELISA assay. The results showed no significant elevation of CRP or other inflammatory markers at any time point. This suggests that conventional orthodontic treatment is not associated with a systemic immune response (MacLaine et al., 2010).

Several clinical and biochemical investigations were also conducted to determine the relationship of inflammatory marker (CRP) levels with obesity. Some studied the association of circulating hs-CRP levels in the presence of obesity with diabetes mellitus in non-obese/nondiabetic, obese/nondiabetic, non-obese/diabetic and obese/diabetic groups. They reported a significant association of serum hs-CRP levels with waist circumference and fasting blood sugar levels (Ebrahimi et al., 2016). In another study the serum concentrations of CRP in obese, overweight and normal weight breast cancer women, higher levels of circulating CRP were observed in obese patients in comparison to normal weight and levels were significantly correlated with BMI and waist-to-hip ratio (Babaei et al., 2015). Additionally, Rose and co-workers evaluated the concentrations of CRP in blood after weight loss in morbidly obese patients (BMI values of >40 $kg/m^2$) exposed to intensive weight reduction. Initially blood showed significantly high levels of CRP compared with the healthy individuals; however, with about 9.4% reduction in body weight, a significant reduction in the concentration of CRP was observed (Rosc et al., 2015).

## 1.2.6 Myeloperoxidase

The recruitment and activation of polymorphonuclear neutrophils (PMN) constitute key events in inflammation and are characterized by the liberation of intracellular granules (Bogaard et al., 2010). Azurophilic granules of PMNs contain the enzyme myeloperoxidase (MPO), which is included in the host-derived group of compounds that are important for the tissue defence promoted by PMNs (Bernabé et al., 2005).

MPO activity is directly correlated to the number of tissue PMNs (Cao and Smith, 1989) meaning that MPO activity, not its quantity, is used in inflammation studies to determine the number of PMNs (Lamster et al., 1985). In patients with coronary artery disease, acute myocardial infarction and unstable angina it is possible to identify increased levels of circulating MPO (Baldus et al., 2003, Brennan and Hazen, 2003), which indicates a potentially adverse outcome (Tang et al., 2011). MPO plasma levels are also predictive of the outcome for patients in the early stages of the onset of chest pain (Rudolph et al., 2011, Nicholls et al., 2011) and present a correlation with impaired left-ventricular function (Rudolph et al., 2007).

A useful method for the monitoring of periodontal inflammation is the analysis of MPO enzymatic activity in saliva or GCF (Cao and Smith, 1989, Yamalik et al., 2000, Marcaccini et al., 2010). This is particularly interesting because sample collection is not invasive, and the determination method is simple and accessible for standard laboratories (Marcaccini et al., 2010). Various dental studies of the use of MPO in GCF show that MPO is greater in the GCF collected from inflamed sites (Cao and Smith, 1989, Kaner et al., 2006).

Since MPO levels found in the GCF represent the extent of PMN infiltration, determining MPO might prove a valuable technique in assessments of the degree of tissue inflammation; a particularly interesting development for the application of orthodontic force, among other areas. Following the application of this force, PMNs migrate into the tissue to then extravagate in increased numbers into the GCF (Lowney et al., 1995). Changes in the periodontium occur during orthodontic movements, depending on the magnitude, duration and direction of the applied force (Karacay et al., 2007). A further study was conducted in order to establish MPO activity in the GCF and saliva, specifically whole stimulated saliva, of orthodontic patients. Measurements were taken at the baseline, and then 2 hours, and 7 and 14 days following the application of orthodontic force. The

results showed that 2 hours following application, mean MPO activity increased in both the GCF and saliva of orthodontic patients, before then returning to the baseline level at the 7 and 14-day measurement points in both saliva and GCF (Marcaccini et al., 2010). In relation to the degree of dental crowding, MPO activity was measured in GCF and whole saliva during orthodontic alignment phase of patients have severe and minimal crowding, at baseline, 2 hours, 7 and 14 days after activation of orthodontic appliance. The results showed that the MPO activity was not influenced by the amount of dental crowding, therefore the two groups of moderate and severe crowding showed increases in salivary and GCF levels of MPO after 2 hours in comparison to baseline and remained elevated till the seventh day to return back to the baseline levels after 14 days (Navarro-Palacios et al., 2014).

There is evidence of association of MPO with obesity. Several studies were conducted in this field; the circulating concentrations of MPO were significantly increased in severely obese compared to controls, which remain elevated even after bariatric surgery and weight loss (Nijhuis et al., 2009). Similarly, circulating MPO in obese individuals, with or without systemic inflammation and potential cardiovascular risk, increased in obese subjects with hs-CRP above 3 mg/l (a biomarker for inflammation and cardiovascular risk) (Borato et al., 2016). More recently higher levels of MPO were remarkably observed in obese children in comparison to controls (Varma et al., 2016, Marcovecchio et al., 2016).

### 1.2.7 Matrix metalloproteinase

Matrix Metalloproteinases (MMPs) are a family of endopeptideases that are zinc-dependent and contain more than 25 enzymes. These play a key role in regulating various biological processes and are key to PDL remodelling in terms of both physiological and pathological conditions (Stamenkovic, 2003). These 25 enzymes can be divided into subfamilies according to their primary structures; cell localization and substrate specificity, including collagenases (MMP1, MMP8 and MMP13) disintegrate native fibrillar collagens. Gelatinases MMP2 and MMP9 are known to cleave denatured collagen (gelatin), meaning that they complement collagenases where the degradation of fibrillar collagens occurs (Pilcher et al., 1999, Quasnichka et al., 2005); the same can be said for stromelysins (MMP3, MMP10 and MMP11), membrane-type MMPs (MMP14, MMP15, MMP16 and MMP17), and a selection of miscellaneous MMPs (Jabłońska-Trypuć et al., 2016).

Collagenases cleave interstitial collagens I, II, and III at a specific site and also cleave other extra cellular matrix (ECM) and non-ECM molecules. Fragments of collagen are then degraded by gelatinases (Raffetto and Khalil, 2008). MMP8 is mainly produced by the polymorphonuclear leucocytes, but the epithelial cells and fibroblasts also have a role to play in its production (Mäntylä et al., 2003, Ingman et al., 2005).

Gelatinases (MMP2 and MMP9) are responsible for type IV collagen degradation, vasculature remodelling, angiogenesis, inflammation and atherosclerotic plaque rupture (Jaiswal et al., 2011). They are secreted by several vascular cell types, including endothelial cells, fibroblasts and myofibroblasts, monocyte derived macrophages and local tissue macrophages (Bourboulia and Stetler-Stevenson, 2010). MMP2 is constitutively expressed on cell surface, while MMP9 is stored in secretory granules in different cell types and it is inducible by exogenous stimuli, such as cytokines, growth factors or altered cell–matrix contacts (Amălinei et al., 2007, Bourboulia and Stetler-Stevenson, 2010).

MMPs are secreted as inactive proenzymes, and these can later be activated by proteolytic processing in the ECM (Snoek and Von den Hoff, 2005). Their activity is coordinated by the tissue inhibitors of metalloproteinases (TIMPs) (Verstappen and Von den Hoff, 2006). MMPs and TIMPs are crucial for the physiological remodelling of the periodontium and responses to forces of mechanical stimuli during orthodontic procedures (Ingman et al., 2005). The use of synthetic MMP inhibitors has, in a number of studies, been shown to diminish OTM (Holliday et al., 2003, Bildt et al., 2007).

A variety of research studies focus on both in vivo and in vitro levels of MMPs in gingiva (Redlich et al., 2001), gingival and periodontal fibroblasts, as well as in GCF (Miyoshi et al., 2001, Apajalahti et al., 2003) in relation to orthodontic forces. Within the periodontium, a careful alteration process of the ECM is essential during orthodontic treatment. This allows for both tooth movement and the preservation of periodontium functional integrity (Junior et al., 2011).

Levels of MMP8 in GCF have been studied for treatment with fixed orthodontic appliances, identifying a relationship between levels and stages of the treatment under the condition of a good control of the bacterial plaque (Apajalahti et al., 2003). A relationship is also present with the pain reported by numerous patients within the first hours of the insertion of the orthodontic device (Avellan et al., 2005). Both of these links can be explained by way of the participation of MMP8 in the generation of PDL remodelling during dental movement. Other authors engaged in comparable research sustain that MMPs

are involved in the regulation of bone remodelling in the marginal periodontium, during which ECM components (including collagen) are broken down and removed, before new components are then synthesized and deposited (Holliday et al., 2003).

Experiments on laboratory rats concerning MMP production in the PDL during orthodontic movement reveal that MMP8 is produced first, by cells at the cemental surface and, second by osteocytes in alveolar bone (Takahashi et al., 2003). However, MMP8 is not produced in other PDL cells. Furthermore, it has been shown that hypertrophied gingiva during orthodontic treatment may be triggered by MMP8 accumulation in GCF; this can even occur in the absence of plaque accumulation or gingivitis. This can be observed in a study that measured the levels of MMP8 in GCF in relation to gingival overgrowth (GO) during orthodontic treatment. GO is a common result of mechanical stress and periodontal remodelling. Periodontal examination and GCF collection took place 1 hour before treatment and then 1, 4 and 8 hours and weekly after treatment throughout a period of 8 weeks. Researchers found that for patients who did not develop GO MMP8 levels increased during the first 4-8 hours following orthodontic appliance activation, before then returning to their original level. Conversely, in patients with GO, the level of MMP8 in GCF continued to rise. For those cases of GO with inflammation, MMP8 levels were significantly higher than those without, meaning that MMP8 values in GCF show potential as a marker of GO (Surlin et al., 2010). However, another study examined the levels of MMP 1, 2, 3, 7, 8, 12 and 13 in GCF of periodontally compromised teeth 1 week before orthodontic activation and then 1 hour, 24 hours, 7 days, 14 days and 21 days following orthodontic appliance activation. They found that the orthodontic movement of periodontally compromised teeth without active pockets did not result in significant changes in the GCF levels of MMPs. The only significant change was found between the two groups occurred in the MMP 12 levels at 24 hours after orthodontic activation (Almeida et al., 2015).

One specific in vitro study suggests that MMP9 is important for the degradation of collagen matrices since it enables the migration of osteoclast precursors to sites of bone resorption (Ishibashi et al., 2006). As a result, MMP9 may also act as a biomarker for the monitoring of remodelling in periodontal tissue during tooth movement. Mostly, MMP9 is derived from monocytes and macrophages, with its levels in GCF being greater for patients with periodontitis. Consequently, it may act as a biomarker of periodontal disease and help with the early detection of periodontitis (Ingman et al., 1996). Human gingival tissue, GCF

and saliva all exhibit a marked increase in MMP9 activities from PMNs sources (Ingman et al., 1996). A further study measured the influence of orthodontic retainers on periodontal health status biomarkers in GCF and found no relationship between fixed retainers and periodontal disease (Rody Jr et al., 2011). This finding has been confirmed by a series of other studies (Pandis et al., 2007, Booth et al., 2008). However, MMP9 exhibits a significant level of elevation for a higher percentage of sites with visible plaque in the incisor region of the fixed retainer, indicating that a dental plaque may induce the release of MMPs in GCF, regardless of clinical inflammation. Measurements of the levels of MMP3, MMP9 and MMP13 in canine retraction during OTM at different time intervals have found statistically significant fluctuations of MMP3, MMP9 and MMP13 at the compression side during OTM, with levels rising within the first hour of orthodontic force application before decreasing significantly during the following 24 hours; after this period of time, levels increased at a gradual pace (Junior et al., 2011). Finally, in humans MMP levels change in the GCF as a result of the application of orthodontic forces (Apajalahti et al., 2003).

The tissue inhibitors, TIMPs are characterized as slow and tight-binding inhibitors. Mammalian TIMPs are two-domain molecules with N-terminal domains of about 125 amino acids. They also have a smaller C-terminal domain of about 65 residues and each domain is stabilized by three disulfide bonds (Williamson et al., 1990). In isolation, N-terminal domains are capable of establishing a stable native molecule that exhibits inhibitory activity against MMPs (Willenbrock et al., 1993). However, while different TIMPs are known to bind tightly to the majority of MMPs (Liu et al., 1997), reports exist of various dissimilarities in inhibitory properties among different TIMPs. As a result, TIMP2 binds tightly to the zymogen of MMP2 (proMMP-2) to form an important complex for the cell-surface activation of proMMP2 (Nagase, 1998). By comparison, TIMP1 forms a specific complex with proMMP9 (Goldberg et al., 1992), while TIMP4 also binds to the C-terminal domain of proMMP2 (Bigg et al., 1997). TIMP3 is extremely challenging to extract from tissues and studies show that it seems to bind strongly to ECM components (Yang and Hawkes, 1992), making the actions of TIMP3 limited to areas near to the point of synthesis.

In terms of tissue remodelling, TIMPs are able to inhibit MMPs by developing 1:1 enzyme-inhibitor complexes. Numerous studies indicate that TIMPs inhibit cellular invasion, metastasis, tumorigenesis and angiogenesis (Gomez et al., 1997), which can be partially attributed to the inhibition of matrixins. However, TIMPs exhibit a variety of

cellular activities. Additionally, TIMPs have a twofold effect on bone resorption in bone metabolism. They are known to stimulate bone resorption at low concentrations by directly stimulating osteoclasts (Sobue et al., 2001), and reduce bone resorption by inhibiting MMP activity at higher concentrations (Geoffroy et al., 2004).

A longitudinal study investigated changes in cytokines and biomarkers for bone and tissue metabolism in GCF for patients of orthodontic treatment. GCF was collected from tension and compression sites at the baseline before tooth extraction and appliance placement, followed by intervals during orthodontic treatment of 4 hours, 7 days and 42 days after the application of distalising forces to the maxillary canine teeth. Tension sites adjacent to the canines exhibited significant increases in MMP9 and TIMPs 1 and 2 across all of the time points used in the study following force application, while increases in MMP9 were seen at compression sites after a period of 7 days (Grant et al., 2013). Another study compared the circulating levels of MMP8, proMMP2, proMMP9 and total MMP9, the tissue inhibitors TIMP1 and TIMP2, as well as the MMP8/TIMP1, MMP9/TIMP1 and MMP2/TIMP2 ratios in obese and non-obese children and adolescents. The results showed higher circulating levels for MMP8 in obese children and adolescents, but not for MMP9 or MMP2. Lower TIMP1 and higher TIMP2 inhibitors were found in obese groups when compared to non-obese groups (Belo et al., 2009). The same study found no gender differences in the levels of MMP2, MMP8, pro-MMP9, MMP9, TIMP1 and TIMP2 between obese and normal children and adolescents, in line with another study reported no influence of gender differences on the levels of MMPs in healthy adults subjects (Tayebjee et al., 2005).

### 1.2.8 Receptor activator of the nuclear factor kappa-B ligand

The Receptor Activator Nuclear Factor Kappa-B Ligand (RANKL) constitutes a crucial factor in osteoclast formation, fusion, activation and survival (Schoppet et al., 2002), and is produced by osteoblasts, stromal and activated T-cells (Udagawa et al., 1999). It has been shown in the literature to prompt bone resorption and bone loss. Alternate splicing is used to produce two isoforms: first, a type II membrane protein (mRANKL) and second, a soluble molecule (sRANKL) that lacks both cytoplasmic and trans-membrane domains. The membrane-bound protein appears to be the homeostatic form – even though both forms are bioactive. Additionally, sRANKL production indicates the presence of both pathologic

and non-physiological conditions (Nakashima et al., 2000). As articulated in the research, RANKL is mRANKL when expressed by cells of osteoblastic lineage. Alternatively, it forms sRANKL when expressed by activated T-cells (Schoppet et al., 2002). RANKL triggers its specific RANK receptor (situated on the osteoclast and dendritic cells) and its signalling cascade (Yasuda et al., 1999).

When RANKL binds to an osteoclast RANK site, the result is an intracellular expression of various factors associated with the TNF receptor (TRAFs). Additionally, mature osteoclast survival depends on the presence of RANKL, although alternative factors, such as IL-1, have also been shown to promote survival. Where this is absent, osteoclasts are subjected to apoptosis (Wise and King, 2008, Eriksen, 2010).

In contrast to the above, osteoprotegerin (OPG) is a secreted member of the tumor necrosis factor receptor superfamily and is known to negatively control osteoclastogenesis. The binding of OPG to the RANKL competitively constrains the binding of RANKL to RANK preosteoclast sites, prompting the termination of the differentiation process that leads to mature osteoclasts. OPG is also known as an osteoclast inhibitory factor, or more specifically, a soluble decoy receptor produced by osteoblastic cells and the cells of the PDL. It then binds to RANKL and inhibits RANK/RANKL interaction, a process that represents the mainstay of osteoclastogenesis (Kanzaki et al., 2004, Vitovski et al., 2007, Boyce and Xing, 2008) (Figure 1.6). Research shows that osteoclast activity is influenced by the osteoblast through the production of RANKL and OPG. Hence, the RANKL/OPG balancing system is crucial in the regulation of osteoclastogenesis (Trouvin and Goeb, 2010, Bae et al., 2011) (Figure 1.7).

Various in vivo and in vitro studies of PDL cells corroborate the role of the RANK, RANKL and OPG system in osteoclastogenesis (Oshiro et al., 2002, Nakao et al., 2007). When compressive orthodontic force is applied, RANKL upregulation is present and thus prompts the stimulation of a PGE2 pathway. Finally, the initiation of osteoclastic activity takes place, thus prompting bone resorption (Wise and King, 2008). An improved understanding of the RANKL/OPG balancing system could lead to new clinical techniques for the identification of optimal forces in tooth movement (Lin et al., 2007, Meikle, 2007). A randomized clinical pilot study evaluated the expression of RANKL and OPG after the application of elastic separators in a split mouth design study. The study found that RANKL increased while OPG decreased in both the short term (24 hours) and long term (7 days) when compared to the baseline (Barbieri et al., 2012).

Levels of RANKL and OPG were also compared in the GCF of adults and juvenile orthodontic patients in an alternative study. This research found that the levels of RANKL increased after 24 hours while those of OPG decreased in both groups. However, the RANKL/OPG ratio was higher for the juvenile patients and this led to a greater amount of tooth movement in juveniles in comparison to the adults in the study (Kawasaki et al., 2006). Further related research has ascertained the levels of RANKL and OPG in the GCF during OTM at intervals of 0, 1, 24, and 168 hours following the application of a retracting force. It also investigated the effect of compression force on RANKL and OPG production from human PDL cells following a compression force (0, 0.5, 1.0, 2.0, or 3.0 $g/cm^2$ for 48 hours). The results showed significantly higher GCF levels of RANKL, and significantly lower levels of OPG, in the experimental teeth than in the control group at the 24-hour mark. Significant differences also remained absent at the 0, 1 and 168-hour marks (Nishijima et al., 2006).

The expression and concentration of RANKL and OPG in human PDL with orthodontic forces of different magnitudes for 7 days is the subject of another related study. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to measure OPG and RANKL mRNA expressions, while ELISA was used in order to assess the concentrations of OPG and RANKL protein in the compression and tension sides of PDL. The results demonstrated that RANKL expression and concentration increase at the compression side due to the application of orthodontic force. The RANKL/OPG ratio was also significantly higher for the experimental rather than the control teeth. It would seem that these finds thus suggest that RANKL contributes to bone modelling as a response to the placement of orthodontic force in its initial stage (Otero et al., 2016).

A review study showed that OPG levels fall immediately after the application of orthodontic force. This signals the beginning of bone resorption – a key process in the induction of tooth movement. The same review showed that juveniles who exhibit an increased RANKL/OPG ratio in GCF display a faster OTM rate than adults (Kapoor et al., 2014). An alternative study was conducted to establish OPG and sRANKL levels in serum and GCF in relation to root resorption in a rat model. Force was applied using an orthodontic coil spring, before the teeth were then extracted and analysed using micro-computed tomography scanning. The results showed a positive linear correlation in serum between RANKL and degree of root resorption, with OPG concentrations decreasing significantly in severe root resorption. It is possible to state then that the OPG/RANKL

ratio might prove a reliable prognostic factor for degree of root resorption following orthodontic treatment. In GCF, RANKL concentration exhibited a negative correlation with RANKL concentration in serum, while the OPG/RANKL ratio suggests whether the dental root will be protected against external root resorption or not (Tyrovola et al., 2010). A different research paper studied the correlation of salivary levels of sRANKL and OPG with the phases of OTM, namely initial, lag and post lag. Here the findings showed that a significant increase in RANKL and decrease in OPG leads to, overall, an increase in the RANKL/OPG ratio in time following the activation visit. The findings thus indicate that changes in salivary sRANKL and OPG, as well as in their ratios, may be linked to the varying phases evident in OTM (Flórez-Moreno et al., 2013a).

Changes in the cytokines and biomarkers of bone and tissue metabolism in GCF among patients partaking in orthodontic treatment have been successfully examined in one particular longitudinal study. Here GCF was collected from the tension and compression sites at the baseline. This was done before tooth extraction and the placement of the relevant appliance, and following this at intervals during the orthodontic treatment: 4 hours, 7 days, and 42 days after the application of distalising forces to the maxillary canine teeth. The results found that after 42 days the compression sites exhibited increases in RANKL levels (Grant et al., 2013).

The roles played by RANK, RANKL and OPG in periodontal tissue reactions, in response to orthodontic forces, have been investigated in another study. The results showed that RANKL concentrations in GCF increased during OTM, and the ratio of RANKL to OPG concentrations in the GCF was significantly greater than at the control sites. A number of in vivo studies have confirmed the presence of RANKL and RANK in periodontal tissues during the experimental tooth movement of rat molars. They have also shown that when placed under mechanical stress PDL cells may induce osteoclastogenesis via the upregulation of RANKL expression during OTM. As a consequence, it is reasonable to assume that the RANKL⁄RANK⁄OPG system has a key role to play in OTM (Yamaguchi, 2009).

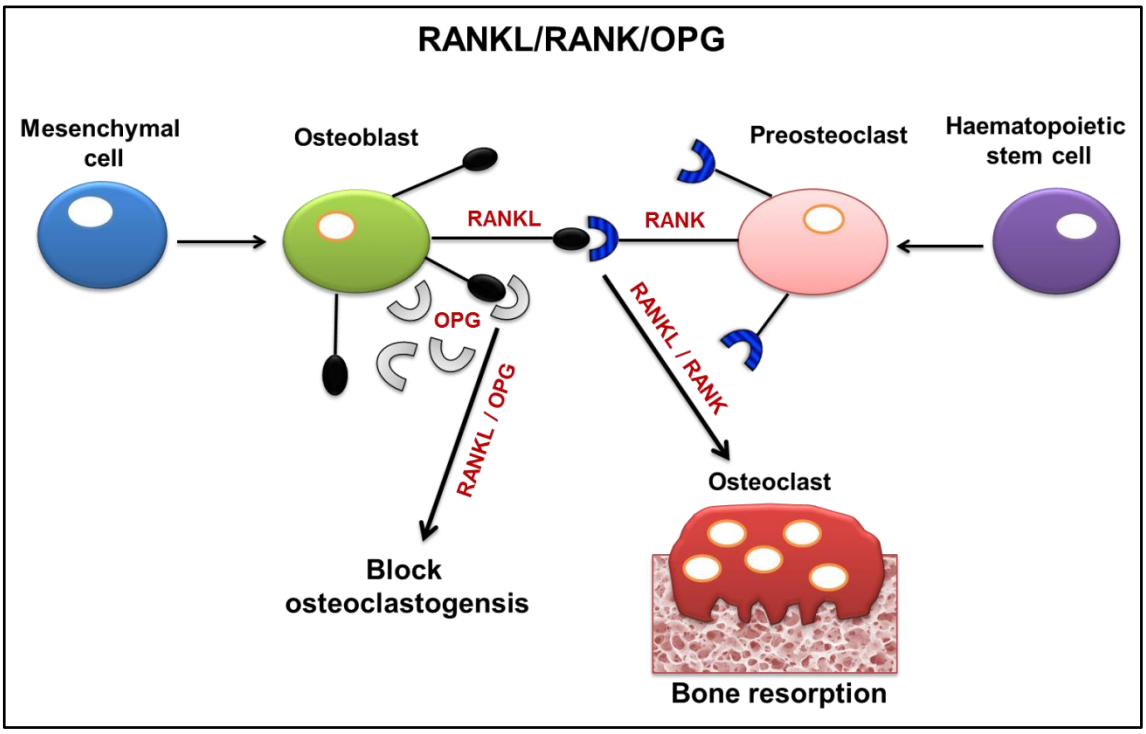



**Figure 1.6** Role of RANKL/RANK/OPG pathway in bone remodelling. Osteoblasts are derived from mesenchymal stem cell. Preosteoclasts are differentiated from hematopoietic stem cells. RANKL-receptor activator of nuclear factor kappa B ligand-produced by osteoblasts binds to RANK-receptor activator of nuclear factor kappa B-on preosteoclasts forming mature osteoclasts; OPG-osteoprotegerin- produced by osteoblasts binds to RANKL and inhibits RANK/RANKL interaction.

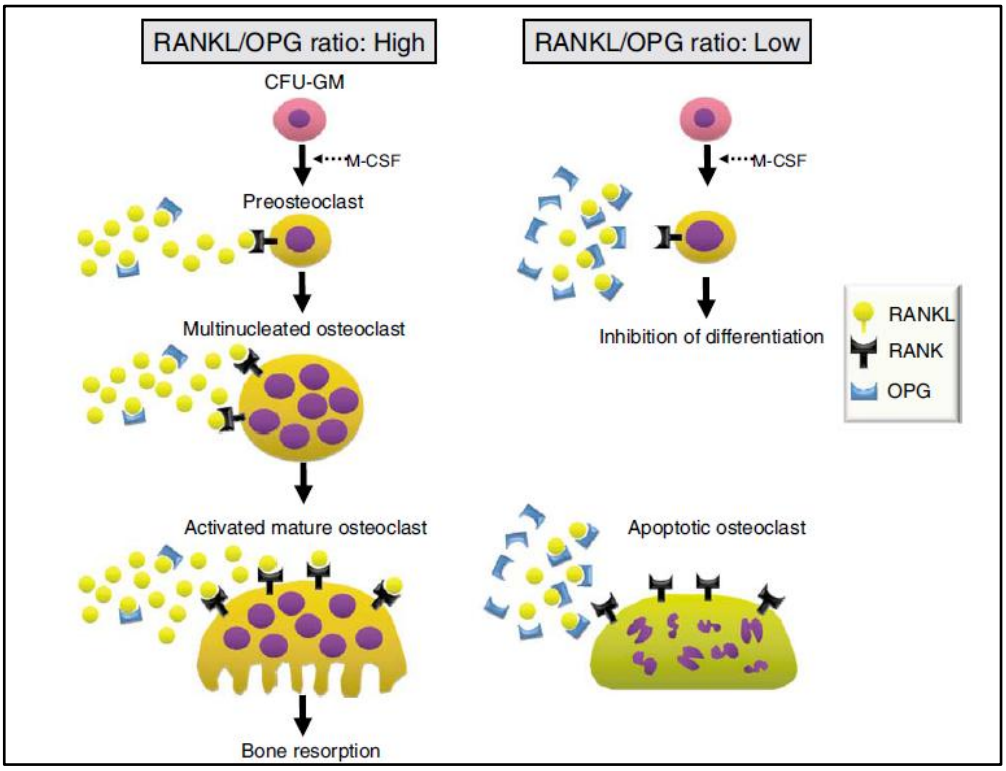



**Figure 1.7** RANKL/OPG ratios. RANKL, receptor activator of nuclear factor kappa B ligand; RANK, receptor activator of nuclear factor kappa B; OPG, osteoprotegerin. Acknowledgments to (Kajiya et al., 2010).

## 1.3 Orthodontic tooth movement

OTM represents the movement of a tooth through alveolar bone due to the application of an appropriate external orthodontic force. This results in strain within the biological system, which forces cellular components to adapt to their new and modified conditions (Henneman et al., 2008). OTM differs from physiological tooth movement, which refers to tooth eruption or drift characterized by the abrupt generation of both compression and tension regions within the PDL (Reitan, 1960). OTM is therefore considered as exaggerated physiological movement may occur at speed or slowly, with this being determined by the physical characteristics of the force being applied, as well as the size and biological response of the PDL (Rygh and Brudvik, 1995). Conversely, physiological tooth movement is consistently a slow process and occurs mainly in the buccal route into the cancellous bone, aiming at growth in the cortical bone.

Two interconnected OTM processes, namely deflection or bending of alveolar bone and remodelling of the PDL, alveolar bone, dental pulp and gingival tissue, on which the applied force causes a compression on one side with an equivalent stretching force on the opposite side of the alveolar bone with the PDL (Dolce et al., 1996). Mechanical loading is also known to modify the vascularity of periodontal tissue and its blood flow. This in turn, leads to the local synthesis and release of numerous neurotransmitter molecules, cytokines, growth factors, and colony-stimulating factors; all of which are involved in the maturation of immune cells and arachidonic acid metabolites. For a range of cell types, cellular responses are prompted both in and around the teeth by the liberated molecules, thus generating a constructive microenvironment for the deposition or resorption of tissue (Sandy et al., 1993). Thus, OTM unlike other inflammatory conditions of PDL, such as periodontitis, in which the inflammatory condition is characterised by alveolar bone destruction and loss of marginal PDL result from imbalance between pro- and anti-inflammatory signals in response to the accumulation of dental plaque adjacent to the gingival margin (Socransky et al., 1984).

Numerous factors influence the success of orthodontic treatment, such as periodontal health, oral hygiene and orthodontic forces. New methods for the quickening of OTM have been pursued by clinicians for a number of reasons: shorten treatment times, reduce adverse effects, such as pain, discomfort, dental caries, periodontal disease, and minimize iatrogenic damage, such as root resorption (Dolce et al., 2002, Krishnan and

Davidovitch, 2006a, Cardaropoli and Gaveglio, 2007, Zainal Ariffin et al., 2011, Andrade et al., 2012).

### 1.3.1 Phases of OTM

Three phases of tooth movement have been identified by Burstone (1962) (Figure 1.8). The first initial phase occurs when an orthodontic force is applied to the tooth, resulting in tooth displacement and evidence of rapid movement in the space of the PDL. This phase comprises both cellular and tissue reactions, and includes the recruitment of osteoclasts and osteoblasts on the compression and tension sides.

The second lag phase involves hyalinization at the compression site of the PDL, which takes place due to the distortion of PDL fibres. This in turn affects blood flow, leading to little or no tooth movement until the complete removal of necrotic tissue has been accomplished. On the tension side, pre-osteoblast cells proliferate and migrate towards the alveolar bone surface to produce new bone matrix (osteoid).

In the final postlag phase tooth movement resumes. Here the pressure side exhibits indirect bone resorption by the removal of the ischematic bone close to the hyalinization, followed by direct bone resorption in the remodelling process, while bone deposition occurs simultaneously on the tension side (Melsen, 1999).

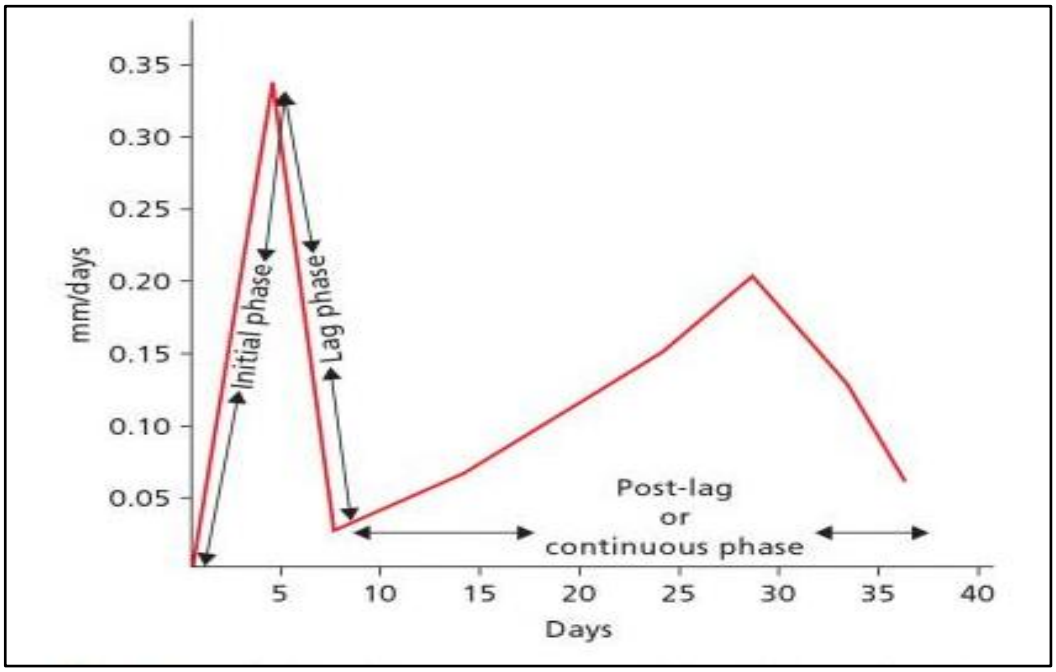



**Figure 1.8** Rate of tooth movement plotted against number of days following application of continuous force. Acknowledgments to (Burstone, 1962).

### 1.3.2 Cellular and molecular changes in OTM

During OTM regions of compression and tension develop within the PDL. The compression region is pressed by the orthodontic appliance in the direction of the force, which leads to the deformation of blood vessels and disruption in the arrangement of the tissue surrounding the teeth. PDL cells undergo metabolic changes due to hypoxia and decreased nutrient levels, with cells coming to rely on anaerobic glycolysis in hypoxic conditions. Many of the enzymes involved in anaerobic metabolism can act as potential markers; for example, the lactate dehydrogenase molecule accumulates during anaerobic metabolism (Passarella et al., 2008). Hyalinization is frequently triggered by mechanical force, often leading to PDL necrosis and subsequent bone resorption. Hyalinization occurs in cell-free areas of the PDL, at these sites the normal tissue architecture and staining characteristics of the collagen have been lost in the processed histological material. A selection of studies report distortions in the arrangement of normal periodontal fibres, numerous cell fragments (debris), areas of degraded matrix interspersed between intact collagen fibrils, and, in some cases, the presence of pyknotic nuclei in the hyalinization areas (Bonafe‐Oliveira et al., 2003, von Böhl et al., 2004, Kitase et al., 2009). Following OTM, a response is prompted from the tissue, yet bone repair only occurs at the compression region when the magnitude of the force decreases. During the early stages of OTM, the markers for bone remodelling can be analyzed through the measurement of both osteoclastic and osteoblastic activities at the compression regions (Faltin et al., 2001, Bonafe‐Oliveira et al., 2003, Casa et al., 2006, Andrade et al., 2012).

In the tension region, the forces applied by braces during orthodontic treatment lead to the formation of new bone. Local precursor cells, or mesenchymal stem cells, are differentiated from osteoblasts, while mature osteoblasts move on to form osteoids and a process of mineralization ensues. In addition to this, endothelial nitric oxide synthase (eNOS) has been shown to mediate bone formation in the area of tension, which thus suggests that eNOS could provide a useful marker for osteoblastic activity (Sprogar et al., 2008, Tan et al., 2009, Andrade et al., 2012). A number of research papers and studies have investigated the link between enzyme profiles and alveolar bone formation at the sites of tension, while alkaline phosphatase has been identified as another biochemical marker that has been proven to be valuable and reliable during osteoblastic activity (Ariffin et al., 2010, Yazid et al., 2010).

### 1.3.3. How do teeth move under orthodontic force?

OTM can cause successive responses in both the periodontal tissue and alveolar bone. At the start of orthodontic treatment, acute inflammation takes place and is characterized by the dilatation of blood vessels in the supporting periodontal tissue. Plasma and leucocytes travel out to produce various local and systemic biochemical signal molecules that evoke the synthesis and secretion of various substances, such as prostaglandin, growth factors and cytokines. Acute inflammation lasts for 1-2 days before chronic inflammation begins. During this phase fibroblasts, endothelial cells, osteoblasts, and alveolar bone marrow cells proliferate and this continues until the next orthodontic treatment appointment, after which acute inflammation returns due to activation by tooth-moving orthodontic appliances (Krishnan and Davidovitch, 2006a). The orthodontic forces bend the alveolar bone that compress and stretch the PDL, leading to alterations in the electrical neutrality of the alveolar bone. The compressed PDL then becomes the site of intense bone resorption, while stretched PDL areas interface with active osteogenesis sites (Krishnan and Davidovitch, 2009).

In biochemical terms, the forces used in OTM lead to an initial increase in levels of bone-resorptive mediators and the associated receptors, namely IL-1β, IL-8, RANKL, and TNF-α; this occurs as early as 1 minute (Dudic et al., 2006) or 1 hour into the procedure (Karacay et al., 2007), and peaks after 24 hours (Sari and Uçar, 2007, Luppanapornlarp et al., 2010, Kaya et al., 2011, Alikhani et al., 2013, Kuroki et al., 2014). These mediators slowly decrease to the baseline at the following observation point: 48 hours, 168 hours, 14 days and 21 days (Karacay et al., 2007, Sari and Uçar, 2007). Conversely, bone-forming mediators, such as OPG, exhibit an immediate decline on the application of orthodontic force on the site of retraction after 1 hour (Toygar et al., 2008, Tuncer et al., 2013), and at 24 hours (Toygar et al., 2008, Barbieri et al., 2012). Furthermore, during the acute inflammatory phase, pain can be noted due to an increased concentration of inflammatory mediators. In particular, these include cytokines and prostaglandins in the GCF of the moving teeth (Krishnan and Davidovitch, 2015).

Various studies compare the compression and tension sites show a decrease in OPG by 24 hours at the compression side (Barbieri et al., 2012), as well as an increase in RANK and TGF-β1 after 7 days (Nishijima et al., 2006, Barbieri et al., 2012). Various other mediators show temporal variations on the compression side, with IL-1β increasing as soon

as 1 minute (Dudic et al., 2006) or after 4 hours (Grant et al., 2013); RANKL increasing after 24 hours in both juveniles and adults (Kawasaki et al., 2006) or after 42 days (Grant et al., 2013); and IL-8 after 4 hours (Grant et al., 2013) or after 10 days (Tuncer et al., 2005). In contrast, the tension site has been shown in various studies to demonstrate an appreciable increase in TNF-α (Grant et al., 2013) and other bone-resorbing mediators, such as IL- 1β, PGE2 and IL-8. However, this rise is shown to have occurred earlier than for compression and, across all of the observation points, with levels higher than those seen at the compression side (Tuncer et al., 2005, Grant et al., 2013).

### 1.3.4 Bone remodelling with OTM

Bone growth, adaptation and turnover occur by way of two key mechanisms: modelling and remodelling. Bone modelling is characterized by independent processes of bone resorption by osteoclastic activity and bone formation by osteoblastic activity result in changes in the size and/or shape of bone. Bone remodelling is characterized by a constant balance of osteoclast induced bone resorption and osteoblast induced bone formation, which goes on to replace the existed bone at the same site (Graber et al., 2011). This process occurs across several stages: the initiation of osteoclast formation; bone resorption mediated by the same cells; reversal period which encompasses bone formation mediated by osteoblasts; and finally, matrix mineralization (Nanda and Kapila, 2010) (Figure 1.9).

In the process of bone remodelling the early steps, namely the initiation of osteoclast formation, require the differentiation and activation of osteoclast forerunners to form osteoclasts. The latter of these are then responsible for the beginning of bone resorption process. This resorption is later followed by bone formation and the number of sites that enter into the bone formation phase, merged with the rate of resorption, will prove decisive in overall turnover (Eriksen et al., 1986, Charles et al., 1987).

Bone resorption constitutes the second step in this process and this occurs when the bone surface is prepared. This preparation stage involves the removal of the surface's un-mineralized osteoid, which then lines it and produce various proteolytic enzymes, including MMPs, collagenase and gelatinase. Osteoclasts are known to recognize extracellular bone matrix proteins through cell surface integrin adhesion molecules: examples of such proteins include osteopontin. The osteoclast is then stimulated at the surface of the mineralized bone

and it is believed that this process is mediated through the osteoblast via local factors or cell-to-cell contacts (Fuller et al., 1991).

Osteoclasts are able to resorb the surface of the bone via the production of hydrogen ions – these change the pH balance and dissolve any inorganic elements. Proteolytic enzymes then come into play by degrading the organic components. The organic matrix largely comprises collagen type I (90% of the protein in bone) and is degraded by the lysosomal cysteine proteinases cathepsin B, L, and K, which work in conjunction with the MMPs, collagenase and gelatinase B. Osteoclast activity stops due to apoptosis, also known as programmed cell death, and this implies that the process by which the osteoclast life span is regulated may represent another important determinant in bone metabolism.

Approximately 9 days after resorption beings to take place – at which point the osteoclast has resorbed the bone surface to its maximum depth – a reversal process begins. While, as stated above, osteoclastic activity is reduced by apoptosis, it is also known that increased calcium levels present in the sub-cellular space also impede resorption. Various substances released from the bone matrix during resorption have also been shown to prompt osteoclast deactivation.

Bone formation is thus the final point in a long process and series of events, including the proliferation of primitive mesenchymal cells, the formation of preosteoblasts, matrix formation and mineralization. Osteoblasts meet on the base of the resorption cavity in order to produce osteoid. Local factors such as the transforming growth factor (TGF) and the exposed type I collagen attract cells towards the resorptive defect. The local proliferation of osteoblast precursors is then influenced by TGF, as well as the insulin-like growth factors (IGF) I and II, fibroblast growth factor (FGF) and platelet derived growth factor (PDGF).

Osteoid mineralisation begins 13 days later at a rate of approximately 1 μm per day. Over the course of 120 to 180 days the bone formation process then takes place and only finishes once the cavity is filled (Eriksen et al., 1986).

Overall by virtue of being acutely sensitive and responsive to applied mechanical loads, osteocyte cells are key participants in this process. Their network of cellular projections facilitates communication with neighbouring osteocytes, alveolar bone surface-lining cells, and bone marrow cavity cells. Osteoblasts maintain direct contact with osteocytes and respond to these signals to initiate appositional changes. Activated osteoblasts send signals to the approaching osteoclasts that encourage these cells to begin

resorption of the alveolar bone. Furthermore, the osteoblasts notify the osteoclasts when to stop this process (Xie et al., 2008).

Growth factors released from the bone through a genetic mechanism for osteoclast activation and suppression regulate the remodelling process, making RANK, RANKL and OPG gene products that control both bone resorption and formation sequences (Nanda and Kapila, 2010). In addition, the local hormones prostaglandins, a product of arachidonic acid metabolism, are chemical agents produced by mammalian cells after cell injury, osteoblasts included. These constitute 20-carbon essential fatty acid molecules and are seen to play a key role in the mediation of the inflammatory response that enables tooth movement (Yamaguchi and Kasai, 2005). A variety of other agents have been identified as affecting bone remodelling and tooth movement, including growth factors (platelet-derived growth factors), the parathyroid hormone (PTH), and interleukins or other cytokines through their stimulation of the production of prostaglandin E2 (Kale et al., 2004).

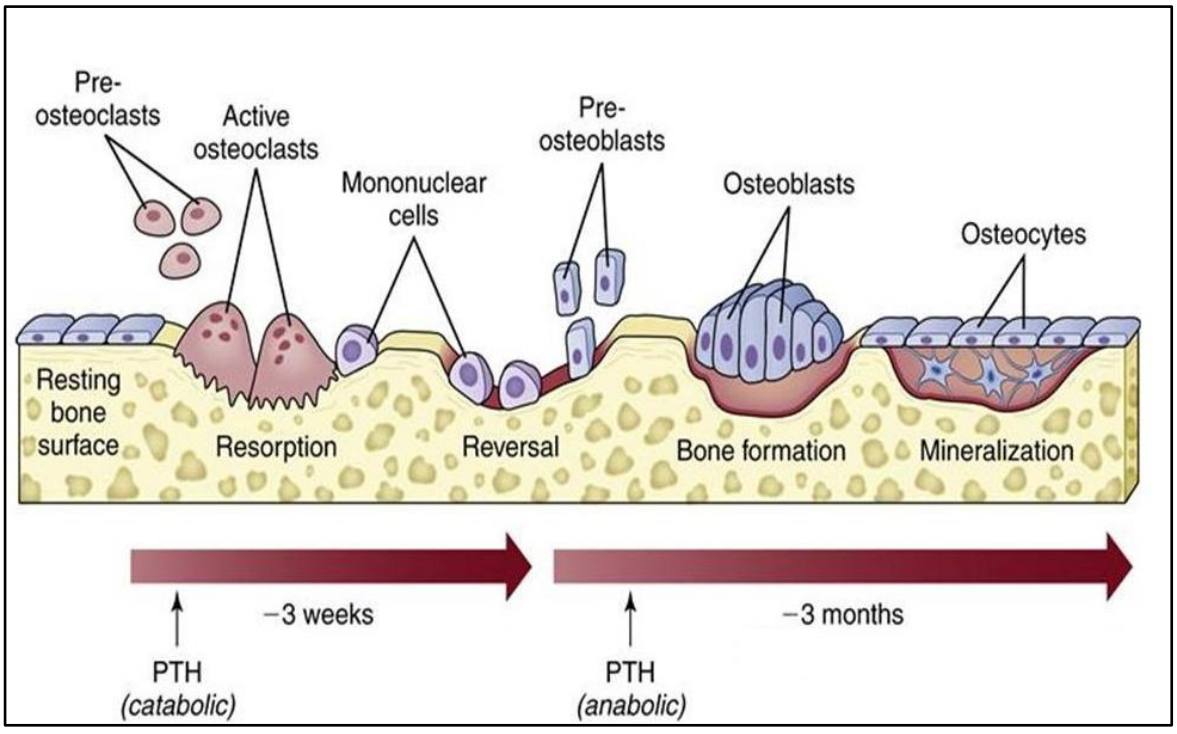



**Figure 1.9** Bone remodelling cycle (initiation of osteoclast formation, bone resorption, reversal process, bone formation and mineralisation). PTH: parathyroid hormone. Acknowledgments to (Garg, 2009).

### 1.3.5 Tissue remodelling with OTM

The area of compression of the periodontium leads to both a narrowing of the PDL and vascular restriction; which can in turn; cause ischaemia, necrosis and vessel degeneration. If the force is strong enough, it may even result in hyalinisation or sterile necrosis. However, if it is possible that the PDL be maintained despite a reduction in the blood supply, cellular activity increases and monocytes are differentiated into fibroblasts and osteoclasts. These fibroblasts and osteoclasts are responsible for the remodelling of both soft and hard tissues. Areas of hyalinisation are lacking blood supply and as a result the host is incapable of recruiting a cellular inflammatory response and, consequently, resorption cannot take place. However, where orthodontic force is distributed in an irregular manner in the PDL adjoining areas of normal periodontium are present. Such areas contribute to a remodelling of the hyalinised areas. In this process, both osteocytes and vascular elements penetrate the hyalinised tissue, and the alveolar bone found in this particular region is removed by underlying resorption (Rygh, 1973).

The tension sites show stretching of the PDL fibres, vascular dilatation and a rise in local vascular activity (Rygh et al., 1986). In the same areas, the first three days see a reduction in the volume of collagen, while vascularisation increases (Crumley, 1964). Collagenase enzymes are produced by mechanical stress, prompting the development of macrophages and fibroblasts (Sandy, 1992). Collagen fibres within the PDL exist in coiled forms. When tooth movement exceeds intrinsic fibre length, new fibres need to be synthesized and incorporated into the ligament proper. Progenitor cells generate osteoblasts via local production within the periodontium (EugeneRoberts and Chase, 1981). After vascular dilatation increased vascular activity can be seen in a particular area. Sheets of densely packed cells are formed on the bone by osteoblasts and the extracellular organic matrix of bone is secreted. Here the components include type I collagen, osteocalcin, osteoponin and osteonectins. Cytokines, proteoglycans and growth regulating factors are also released. (Rygh et al., 1986).

Furthermore, blood vessels are crucial in mediating OTM, with angiogenesis and remodelling of existing blood vessels aiding in adjustments to the new environment created by mechanical forces. It has been reported that orthodontic force application is followed by a preliminary reduction and subsequent increase in the number of PDL blood vessels (Anastasi et al., 2008, Ren et al., 2008). These blood vessels execute a key role in

mechanical force-induced aseptic inflammation, in which they operate as sources for both cytokines and chemokines. Demonstrating a confirmation of the presence of aseptic inflammation, increased expressions of IL-1β, IL-1 receptor, IL-6, IL-6 receptor, IL-8 receptor, IL-11, and TNF-α have been demonstrated in the compressed PDL (Koyama et al., 2008).

### 1.3.6 Rate of tooth movement

The displacement of a tooth per unit time is used to describe the rate of tooth movement and is typically measured in mm per hour, day, week or month. As little as one millimetre per month counts as an acceptable rate of tooth movement (Walther et al., 1994, Graber et al., 2011).

It is normal for all of the teeth involved in a fixed orthodontic appliance to move differently or independently, rendering a quantification of this distance difficult without the use of reference points. Different methods have been used to measure the rate of OTM. One particularly notable method was developed by Little (1975) to measure the linear displacement of anatomical contact points. OTM rate was measured during the alignment stage, with displacement being reduced with time. This method can thus be employed as an indicator for the rate of the alignment achieved and lower incisor stability following orthodontic treatment.

Fixed-appliances orthodontic treatment of moderate to severe malocclusion usually lasts for a year and a half (Tsichlaki et al., 2016), with some variation according to the associated factors (Fisher et al., 2010, Mavreas and Athanasiou, 2008). Recently, both orthodontists and patients have become interested to accelerate tooth movement (Uribe et al., 2014), particularly adult patients who prefer to finish the treatment with the shortest time for social and aesthetic concerns (Rosvall et al., 2009). Additionally, reduced orthodontic treatment duration has significant advantages to decrease the side effects of orthodontic treatment, such as pain, caries, gingival recession and root resorption (Talic, 2011, Segal et al., 2004).

More recently, numerous surgical and nonsurgical adjuncts to orthodontic treatment have been outlined to reduce treatment duration. Surgical techniques include alveolar corticotomy (with or without local augmentation) and various forms of distraction (Uzuner and Darendeliler, 2013). There is some evidence that surgical intervention can increase

rates of tooth movement in the short term (Gkantidis et al., 2014, Hoogeveen et al., 2014); however, discomfort, morbidity, and inconvenience for the patient are likely to be associated with surgery. Among nonsurgical methods, a low-intensity laser irradiation was used in humans during the retraction of upper canines in a 2-month split mouth design study, using mechanical activation in control side and the same mechanical activation with irradiation by a diode laser emitting light on 4 days of each month on the other side. A significant acceleration in the canine retraction by 34% more in lasered side compared to the control was observed, with no evidence of damage to the surrounding bone and tissues (Cruz et al., 2004). One clinical trial was also performed to determine if photobiomodulation reduces the orthodontic alignment time by using a near-infrared light device to irradiate the surface of the cheek 20 or 30 minutes per day or 60 minutes per week with traditional fixed orthodontic appliance. They found that the rate of change in the irregularity was 0.49 and 1.12 mm/week for the control and test groups, respectively, suggesting that photobiomodulation produced remarkable clinical changes in the OTM (Kau et al., 2013). The other non-surgical methods to accelerate OTM include pulsed electromagnetic field, which was investigated using a split mouth design study during canine retraction. The results showed that canines exposed to pulsed electromagnetic fields retraction was 1.57 ± 0.83 mm more than control canines (Showkatbakhsh et al., 2010).

Pharmacological approaches to accelerate tooth movement have also investigated. One study measured the rate of tooth movement with and without submucosal injection of $PGE_1$ during distal movement of canine. The results showed that the rate of canine retraction was almost 1.6-fold on the side of $PGE_1$ injections in comparison to the control side (Yamasaki et al., 1984). Conversely, another study conducted to assess the effect of relaxin -soft tissues remodelling hormone- on the tooth movement and stability in humans. They found that there were no significant differences in the OTM between relaxin and placebo groups (0.08 mm and 0.09 mm, respectively) over the 8-week treatment; and in relapse from week 8-12 (0.07 for relaxin and 0.08 for the placebo) (McGorray et al., 2012).

Although numerous innovations in the design and construction of fixed appliances have been advocated, there is little high-quality evidence to suggest that bracket design or arch wire sequence can significantly influence the speed of tooth movement (Papageorgiou et al., 2014b, Papageorgiou et al., 2014a, Jian et al., 2013). One particular study conducted to compare mandibular teeth alignment using self-ligating and a conventional edgewise orthodontic bracket system, found no difference in the initial rate of mandibular incisor

alignment rate (0.119 mm/day and 0.135 mm/day) respectively, between them (Scott et al., 2008a). Another randomized trial investigated the influence of supplemental vibrational force on the rate of orthodontic tooth alignment with fixed appliances in three groups of patients (1: using removable intraoral vibrational device, 2: using an identical non-functional device, 3: fixed appliance only). No evidence was found to indicate that supplemental vibrational force might significantly increase the rate of initial tooth movement. Similarly, no evidence was found that it might reduce the amount of time needed to achieve final alignment in the three tested groups (210.2, 217.5, 200.7 days), respectively (Woodhouse et al., 2015a). A double blind randomized clinical trial study investigated the effects of low diode laser on the rate of canine retraction on days 1, 3, 7, 30, 33, 37, 60, 63 and 67 using sectional closing coil and found comparable rate of tooth movement in both irradiated and non-irradiated sides at each time point (Dalaie et al., 2015).

The duration of treatment and number of visits were compared using two different fixed orthodontic appliances (self-ligating and conventional brackets). They found that the bracket type did not significantly influence the duration of treatment (21.4 vs. 18.3 months), the number of visits required (17.8 vs. 15.7) and the percentages of PAR score reductions (68.5% vs. 74.3%) (Fleming et al., 2010). This confirmed by the outcomes of another study used same appliances, showed the overall treatment duration (24.5 vs. 23.0 months), number of visits (14.2 vs. 14.5), or overall percentage of reduction in PAR scores (85.2% vs. 83.4%) in the treatment of patients need teeth extraction to fix the crowding (DiBiase et al., 2011).

One in vivo study investigated the effects of caffeine intake on OTM in rats. After a static compressive force was applied for a period of 3 weeks, the degree of tooth movement and its effect on the periodontium were assessed in vitro for groups both with and without caffeine groups. The results showed that in vivo, the caffeine group exhibited a significantly greater rate of tooth movement than the control group. In vitro, caffeine at 0.01 mM significantly heightened the compression-induced expression of RANKL and COX-2, and prostaglandin E2 production in the PDL. They have thus suggested that a daily caffeine intake of a yet-to-be specified dosage may enhance OTM by increasing osteoclastogenesis (Yi et al., 2016).

The rate of orthodontic movement is primarily affected by:

**Force Magnitude**: Tooth movement increases proportionally with force, yet only up to a point. Forces that go beyond this point prompt unnecessary trauma to biological tissues and can prove stressful for the anchorage. The force magnitude applied to an individual tooth depends upon two elements: first, its root surface area and second, the type of tooth movement planned. With regard to this topic, research can be found that specifically cites the optimum force for tooth movement at being around 20-25 $g/cm^2$ of root surface area (Proffit et al., 2014).

**Force Duration**: In general terms, orthodontic force is applied in either an intermittent (removable appliances), interrupted or continuous (fixed appliances) manner. The blood stream begins to show chemical indicates for OTM within a few hours of the force application and then clinical tooth movement takes place. Yet a light continuous force is superior to intermittent force for optimal tooth movement due to the nature of cell biology, that is, a system that remains in a persistently responsive state as opposed to a shifting state (Samuels et al., 1998).

**Age**: Both tooth movement and biologic responses to orthodontic treatment are slower in adults than in adolescents and children. This can be attributed to the fact that adult PDL is much less cellular and the density of their alveolar bone much greater (Walther et al., 1994). A higher RANKL/OPG ratio in GCF for juveniles can be used to explain the greater velocity and amount of OTM in juveniles compared to that for adults (Kawasaki et al., 2006).

**Occlusal interlock:** Normal occlusion in orthodontics has developed as the result of a particular concept that requires teeth have a specific arrangement: an intra-arch relationship and inter-arch to opposing arches. Well-aligned dental arches with normal labial and buccal over jet, overbite and have a normal antero-posterior relationship between maxillary and mandibular arches constitute normal occlusion. A good buccal occlusion may act to resist tooth movement (Gray, 2004).

**Individual variation:** A series of factors may be responsible for the variations that occur in the rate of tooth movement, including rate of body growth, metabolism, bone density and PDL turnover (Kusy and Tulloch, 1986, Pilon et al., 1996).

**Drugs and chemical agents:** Extensive research has been conducted into the accelerative or preventive effects of various drugs and chemical agents on OTM, and it has been shown that the rate of tooth movement can be altered by the application of certain agents either locally or systemically. Prostaglandin, vitamin D and corticosteroids constitute promoter

agents and they are responsible for the acceleration of the required tooth movement via the augmentation of the bone resorption process. Suppressor agents, such as nonsteroidal anti-inflammatory drugs, and bisphosphonates lessen tooth movement by either reducing bone resorption or enhancing bone formation (Valiathan and Dhar, 2006, Bartzela et al., 2009).

## 1.4 Human body fluids as a source of biomarkers

The human body comprises a wide variety of fluids such as blood, urine, saliva, GCF, tears and sweat, all of which possess an expansive selection of proteins. These fluids fill organs or body cavities after circulating through the body, before being secreted or excreted. Not only are they key to overall health and well-being, but they also offer clues to the body's biological processes and its functions. For example, blood, urine, and cerebrospinal fluid, are applied clinically in the examining of human health and the diagnosis of diseases (Loo et al., 2010). Serum and plasma, which are found in the blood, are particularly popular in clinical testing since they surround all tissues and organs, possible gathering the byproducts of disease. Varying concentrations of specific plasma have been linked to certain diseases.

In terms of whole saliva, this is recognized as a potential disease biomarker for both discovery and diagnosis. It is readily available from most individuals, can be easily collected, stored and processed, and requires a simple and non-invasive collection process. A compare and contrast exercise for the protein compositions of salivary and plasma fluids are thus worthwhile. Significant overlaps in protein content between saliva and plasma may present saliva as a diagnostic and an alternative to blood tests (Loo et al., 2010). This area has made great progress since the mid-2000s, when advancements began to take place in salivary testing for health and disease in both oral and systemic terms (Schipper et al., 2007, Genco, 2012, Wong, 2012).

Applied force causes local inflammation and greater capillary permeability in paradental tissues during orthodontic treatment. Since GCF occurs closer to the sites of these activities and is less likely to be diluted, it has a better diagnostic potential than saliva for the markers of these activities (Rody Jr et al., 2011). The first studies undertaken into GCF were aimed at bettering clinical judgment, and the prevention and treatment of periodontitis. It was only when our understanding of humeral immune responses, genomics and proteomics improved that the potential applications of GCF and saliva analyses began to be discussed. If the immune and inflammatory responses of an individual to certain

stimuli can be measured through studies of oral fluids, then the results may allow for an assessment of the risk of the individual both orally, and in terms of other systemic conditions (Genco, 2012, Giannobile, 2012).

### 1.4.1 Gingival crevicular fluid

GCF is an exudate found in the gingival sulcus, between the tooth and marginal gingiva. In the healthy sulcus it is a transudate of interstitial fluid and is present in minute amounts. GCF is released into the crevicular sulcus at a flow rate of close to 3 µl/hour (Perinetti et al., 2013), and under stimulated or inflamed conditions reflects the concentration of metabolites in the serum, increasing in volume with the severity of the inflammation up to 44 µl/hour.

The result of increased capillary fluid filtration in comparison to lymphatic uptake leads to fluid accumulation as oedema and/or leaves the area as GCF. It largely comprises serum components, inflammatory cells, connective tissue, epithelium and microbial flora established in the gingival sulcus and exhibits defence activities by flushing carbon particles and bacteria from the sulcus, while its antimicrobial properties and antibodies improve inflammation resistance (Goodson, 2003, Griffiths, 2003).

#### 1.4.1.1 Composition of GCF

The main origin of the aqueous portion of GCF is serum, yet its composition can be modified to a great extent by the products and microorganisms present in its pathway from the gingival tissue through to the sulcus. In general, it comprises cellular components that include bacteria, desquamated epithelial cells and leukocytes that pass through sulcular epithelium, such as PMNs, lymphocytes, monocytes and erythrocytes. These cells originate from blood vessels in the gingival connective tissues as a result of the stimulation of cells and metabolic products in plaque close to the gingival sulcus (Kavadia-Tsatala et al., 2001).

Granulocytes form about 70-80% of GCF cells, while monocytes and macrophages account for around 10-20% and 5% each for mast cells and T-lymphocytes. Together with organic compounds such as carbohydrates, lipids and protein, electrolytes like calcium, sodium, potassium, fluoride and magnesium are also present. Certain levels may be higher in GCF; with glucose concentration 3-4 time that of serum, for example; while protein levels are much lower than in serum. Metabolic and bacterial products such as lactic acid,

hydroxyl proline, urea, endotoxins, cytotoxic substances, prostaglandins, antibacterial factor and hydrogen sulphide have also been shown to be present (Zia et al., 2011).

Other components of GCF are enzyme and enzyme inhibitors such as acid phosphatase, pyrophosphatase, alkaline phosphatase, lysozyme, B-glucoronidase, hyaluronidase and proteolytic enzymes like mammalian proteinases that includes cathepsin D, cathepsin G, elastase, plasminogen activators, bacterial proteinases (endo and exopeptidases, collagenase and lactic dehydrogenase serum proteinase inhibitors like alpha 2- macroglobulin, alpha 1- antichymotrypsin, alpha 1- antitrypsin (Zia et al., 2011).

### 1.4.1.2 Rationale for the study of GCF

The study of GCF was first introduced and pursued in the hope of improving clinical diagnosis, prevention and treatment for gingivitis and periodontal disease. Early studies focused on the amount of GCF and its correlation with clinical and histological measures of inflammation. To summarize, cross-sectional and longitudinal studies find that the amount of GCF is positively correlated to clinical indices of gingival inflammation, while correlation between amount of GCF and histological evidence of inflammation prove weaker and at times discordant (Cimasoni, 1982).

Early efforts evaluate the differences among GCF constituents linked to healthy versus periodontal-compromised teeth or sites, and various studies indicate that levels of GCF constituents reflect both local events and a donor's overall systemic response (Champagne et al., 2003). More recently, improved knowledge of humoral immune responses, genomics and proteomics has broadened the prospective applications of GCF analysis. It has been suggested that if an individual's immune and inflammatory responses to stimuli can be measured and assessed to calculate periodontitis, then via careful analysis an assessment of the relative risks for an individual of developing other conditions could also be made, including diabetes mellitus, cardiovascular disease, HIV and hepatitis (Ebersole, 2003, Lamster and Ahlo, 2007).

### 1.4.1.3 Collection of GCF

GCF can be collected using various techniques, such as gingival washing, immune-magnetic beads, micropipettes or capillary tubing, pre-weighed twisted threads, and absorbent strips of filter paper. The last of these methods is considered less traumatic when

correctly performed and can be applied to the specific site both easily and quickly (Tzannetou et al., 1999).

Intra-crevicular application can be employed, where the strip is inserted into the base of the pocket or extra-crevicular application, where the strip is placed over the crevice region or at its entrance to prevent traumatic irritation and the subsequent simulation of GCF secretion (Newman et al., 2006). No significant differences were found in GCF collected from either site (Griffiths, 2003).

#### 1.4.1.4 GCF collection difficulties

Major sources of GCF contamination constitute blood, plaque and saliva, and hence precise steps should be taken before GCF collection. These include isolation of the teeth with cotton rolls, followed by the gentle removal of the supra gingival plaque and, finally, complete drying of the teeth's surface with an air stream (Griffiths et al., 1992).

In the case of GCF contamination with blood the sample should be discarded, while plaque contamination may affect the determination of the volume. The avoidance of saliva contamination requires sure isolation and alpha-amylase could be used to confirm or disprove contamination by saliva in a sample (Griffiths et al., 1997).

#### 1.4.1.5 Volume of GCF

GCF volume is measured according to the surface area of the strip dampened by the fluid. For a more accurate measurement a staining of the strip can be done, either with ninhydrin, which gives a purple colour as an indicator, or by the systemic administration of 2 g fluorescein 2 hours before GCF collection, followed by a UV-light examination of the stained strip; the latter of these methods is more sensitive to protein staining. The volume of collected GCF can be measured by comparing the strip weight before and after sample collection. However, this is quite a sensitive process due to the small amount of fluid secreted from a healthy crevice. Periotron provides a more reliable method and involves an instrument that measures the volume and composition of the collected sample using an electrical current that is passed through the dampened strip (Golub and Kleinberg, 1976). This ultimate technique is fast and leaves no marked effects on the sample.

Sample evaporation presents the main problem in GCF volume measurement, especially for volumes of less than 1 µl. Since such samples require further investigation,

care should be taken to store each strip in a sealed container to avoid any fluid evaporation that may affect volume measurement. Recovery of the collected GCF is essential, with centrifugal elution demonstrating approximately 100% protein recovery (Cimasoni, 1982). One microliter is the maximum strip capacity and a longer collecting time may lead to the collection of more fluid than allowed for by the maximum capacity limit (Goodson, 2003).

Previous evidence shows that GCF volume might prove a better indicator of gingival inflammation than standard clinical assessments (Griffiths et al., 1992). Given that tissue remodelling incident to OTM is elicited by an inflammatory process (Krishnan and Davidovitch, 2006b), it is hypothesized that the volume of GCF production will reflect these tissue changes (Griffiths, 2003). Additionally, the clinical recording of periodontal conditions may exclude the idea that, due to plaque accumulation, gingival inflammation is responsible for an increase in GCF production rather than tooth movement. However, conflicting results are reported in the literature, with studies showing both increased and unchanged GCF volumes incident to OTM (Perinetti et al., 2013).

Across numerous studies, very little or no statistically significant changes in GCF volume incident to OTM has been recorded. The changes that are seen are generally ascribed to clinical or subclinical inflammation following the placement of a fixed orthodontic appliance. Some such studies have compared the volume of GCF during distalisation of the maxillary canine in split-mouth designs in adult and juvenile groups across 4 time points. They found no significant difference between moved and non-moved teeth at each time point or over time within the test and control teeth for both the adult and juvenile groups (Kawasaki et al., 2006, Dilsiz et al., 2010). Comparable results are cited by further study that measure GCF volume at different time points using varying orthodontic force (Luppanapornlarp et al., 2010). A final study notes that GCF volume increase mainly results from subclinical periodontal inflammation following fixed appliance placement, rather than from OTM (Drummond et al., 2011).

#### 1.4.1.6 Flow rate and constituents of GCF

Flow rate describes the movement of fluid from or into the gingival pocket and occurs at approximately a few microliters per hour. It can be measured by monitoring the volume of fluid that crosses a defined boundary at a given time. Its rate and composition can be altered through inflammation induced by OTM or periodontitis. Both a flushing action and an isolation effect are the result of GCF flow, with the substances present in the gingival

sulcus being easily rinsed out by the flushing action. Furthermore, the outward flow of GCF would not allow for saliva to retrograde to the gingival sulcus, resulting in the isolation of the gingival sulcus from the oral cavity (Kavadia-Tsatala et al., 2001).

Under healthy conditions GCF flow rate remains stable over time, while it has been shown to increase with inflammation and decrease with therapy (Goodson, 2003). GCF flow rate was also reported to be increased (Baldwin et al., 1999) or remained with no significant changes (Uematsu et al., 1996a, Miyajima et al., 1990) during orthodontic treatment. A greater quantity of GCF can be collected from adolescents in comparison to adults, regardless of the presence of any stimuli (Ren et al., 2002). Smokers provide a smaller amount of GCF than non-smokers (Hedin et al., 1981).

GCF sampling sequences may affect volume and for healthy sites the fourth and fifth samples have been shown to yield higher volumes of GCF than previous samples due to increased trauma (Chapple et al., 1996). By comparison, protein concentrations remain stable at healthy sites yet increase at inflamed sites with repeated sampling (Curtis et al., 1988).

At this point, it is important to emphasize that GCF constituents are affected by both local and systemic conditions (Ebersole, 2003). Periodontitis is an obvious example of a heterogeneous disease in which both the environment and genes affect severity (Kinane et al., 2005).

### 1.4.1.7 GCF and orthodontic therapy

Studies concerning GCF and orthodontic therapy have both followed and reflected periodontal research, with the mechanical stimuli applied to teeth during orthodontic treatment being expected to affect the amount and constituents of GCF. Histological studies conducted in a guinea pig model demonstrate that orthodontic forces cause localized increased vascular permeability. This increased vascular permeability is similar to that seen in the inflammatory process (Storey, 1973) and appears to support the aforementioned expectation that the mechanical stimuli applied to teeth during orthodontic treatment affect the amount and constituents of GCF.

Numerous studies have examined the relationship between tooth movement and physical, cellular or molecular changes in paradental tissues and modifications in GCF constituents during OTM (Krishnan and Davidovitch, 2009). The results indicate noticeable effects on the quantity and constituents of GCF, together alterations in the haemostatic

condition of the periodontal space – an effect that led to biochemical and cellular changes that re-shape the contour of the alveolar bone (Toms et al., 2002). Less significant changes in GCF were observed in children with good oral hygiene undergoing orthodontic treatment when compared to the control group (Tersin, 1978). Meanwhile, GCF does not constitute an indication for tissue remodelling in orthodontic treatment (Perinetti et al., 2013).

In vitro and in vivo research began to report studies of the measurement of GCF constituents in relation to orthodontic force, with many of these studies being concerned with the identification of GCF markers linked to orthodontic stimuli. Some such studies compare markers at both the experimental and control sites using a split-mouth design study across various time points. Here GCF samples are collected before the application of mechanical stimulus to provide a baseline, and further samples are gathered at a variety of time points after fixed appliance application. The results reveal that, on average, peak levels occur in the markers 1-2 days after the application of force, before returning to the baseline level after 1 week (Giannopoulou et al., 2006, Kawasaki et al., 2006, Karacay et al., 2007). Similar results have been observed for inflammatory biomarkers during the retraction of maxillary canines (Alikhani et al., 2013), while other studies to have compared GCF biomarker levels before, during and after orthodontic treatment reveal no differences between untreated teeth, treated teeth, and teeth with retention (Pender et al., 1994, Griffiths et al., 1998). Others have employed GCF analysis in the testing of the effectiveness of preventive measures against plaque accumulation during OTM (Sköld-Larsson et al., 2003, Paschos et al., 2008), and a selection of alternative studies have measured the levels of ECM proteins in GCF to analyse the presence and levels of root resorption (no resorption, mild and marked) (Mah and Prasad, 2004, Balducci et al., 2007). For instance, a study reported that RANKL/OPG has been significantly increase with root resorption more than 2 mm in comparison to the control samples (George and Evans, 2009). Additionally, age and growth status influence cytokines levels in GCF, which is shown to have an effect on the rate and amount of tooth movement. In one particular study, different mediators were found to increase in different age groups, with IL-6, GM-CSF increasing in juveniles alone and PGE2 increasing in both juveniles and adults (Ren et al., 2002).

## 1.4.2 Saliva

A principal natural media in the oral cavity is saliva, a unique body fluid that continually bathes the area to maintain the healthy tissues of the oral cavity, oropharynx and larynx (Humphrey and Williamson, 2001). Saliva is formed by three pairs of major salivary glands, namely parotid, submandibular and sublingual, and hundreds of minor salivary glands, with some of the GCF being secreted from the gingival sulcus (Welton, 2012). The presence of saliva in the oral cavity is vital for the maintenance of healthy teeth and oral tissues. Its secretion is mediated by the parasympathetic and sympathetic nerve supply, and its type and volume are controlled by the autonomic nervous system (Proctor and Carpenter, 2007). The easy and non-invasive collection and diagnosis of saliva has facilitated extensive research into carrier susceptibility, physiological and pathological changes, and the monitoring levels of hormones, drugs, ions, antibodies and microorganisms.

### 1.4.2.1 Salivary glands

Salivary gland tissue comprises secretory end pieces (acini) and the ductal tree system, with secretion taking place in two stages. First, the acinar cells secrete isotonic primary saliva rich in NaCl. Second, the saliva passes through the striated duct cells where most of NaCl is reabsorbed and K+ is secreted in order to render the saliva more hypotonic as it passes from the ducts towards the mouth (A Catalán et al., 2009, Welton, 2012) (Figure 1.10).

The first major salivary gland is the parotid gland, of which there are two in humans. The largest of the salivary glands, each is located in front of the ear and behind the mandibular ramus. Shaped like a wedge, its base is superficially directed and apex deeply directed, and its secretion is serous or watery in consistency. Second, the submandibular glands are around half the size of the parotid gland, with the superficial part situated between the mandibular body and mylohyoid muscle and its smaller deeper part resting above the mylohyoid muscle in the floor of the mouth. The submandibular glands secrete a mixture of mucous and serous fluids. Finally, the much smaller sublingual glands are about one fifth of the submandibular glands and located in the floor of the mouth, where they produce a predominantly mucous secretion. The minor salivary glands comprise the small buccal, labial, palatal, plalatoglossal and lingual glands found in the oral cavity and they chiefly comprise mucous components. Their viscous secretion is due to a high

concentration of mucin and very low levels of phosphate, with an absence of bicarbonate. Their main ions are sodium, potassium and chloride and they exhibit a fluoride concentration several times that of the major salivary glands (Welton, 2012).

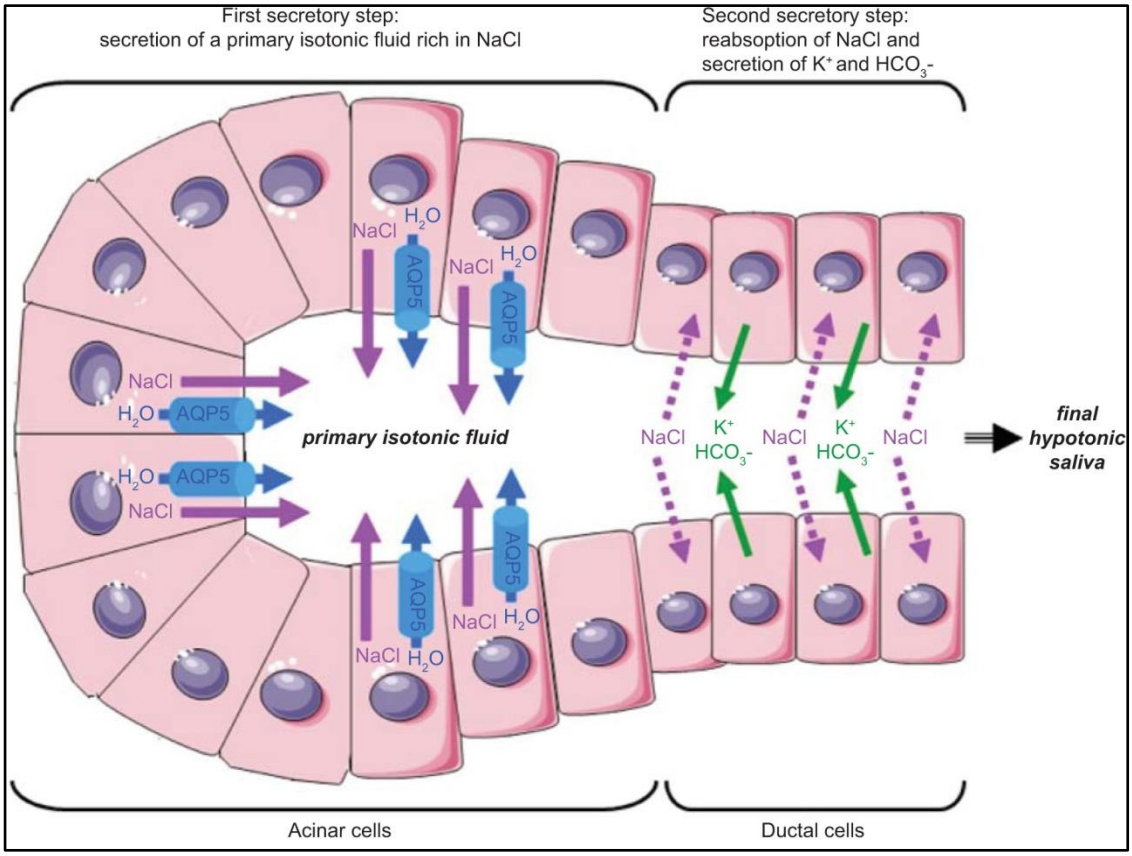



**Figure 1.10** Molecular mechanisms of saliva secretion. Acinar cells secrete a large volume of isotonic-like fluid rich in NaCl. The generation of a trans-epithelial osmotic gradient drives water flow through apical Aquaporin 5 (a small integral membrane protein) and possibly paracellular pathways. Ductal cells, relatively impermeable to water, re-absorb most of the NaCl and secrete K[+] and HCO3[−]. Acknowledgments to (Delporte, 2013).

### 1.4.2.2 Saliva composition

Water forms around 99% of saliva, in addition to various inorganic components such as sodium, chloride, potassium, magnesium, calcium, phosphate and bicarbonate. It also contains organic components such as proteins, including immunoglobulin, enzymes and antimicrobial factors, glycoproteins, albumin, polypeptides and oligopeptides. Glucose and nitrogenous products such as urea and ammonia are also present in saliva (Humphrey and Williamson, 2001, Welton, 2012) (Figure1.11).

Both the type of secreting gland and salivary flow rates can affect saliva composition. For instance, with an increased salivary flow rate the pH and the concentration of proteins, sodium, chloride and bicarbonate increase, whereas levels of magnesium and phosphate decrease. Moreover, saliva composition is affected by duration of stimulation. For instance, with an increase in stimulus duration the concentration of bicarbonate increases and chloride levels decrease. In addition, saliva composition is not affected by the nature of the stimulus, although the stimulus does affect the flow rate, which in turn affects composition. Circadian rhythms are also known to influence composition; for example, sodium and chloride peak in the early morning, while the protein concentration peaks in the late afternoon. In light of these changes, time for saliva collection should be fixed to allow for the standardization of studies. Phosphate concentration in unstimulated saliva is approximately 5-6 mmol/l, which is higher than that of 1 mmol/l in plasma. In contrast, protein concentration in saliva is much lower at only one thirtieth of that in plasma. Urea concentration is about 4 mmol/l, which is slightly lower than that in plasma, and saliva has been shown to contain less calcium (2.5 mmol/l) than in plasma (Dowes, 2012).

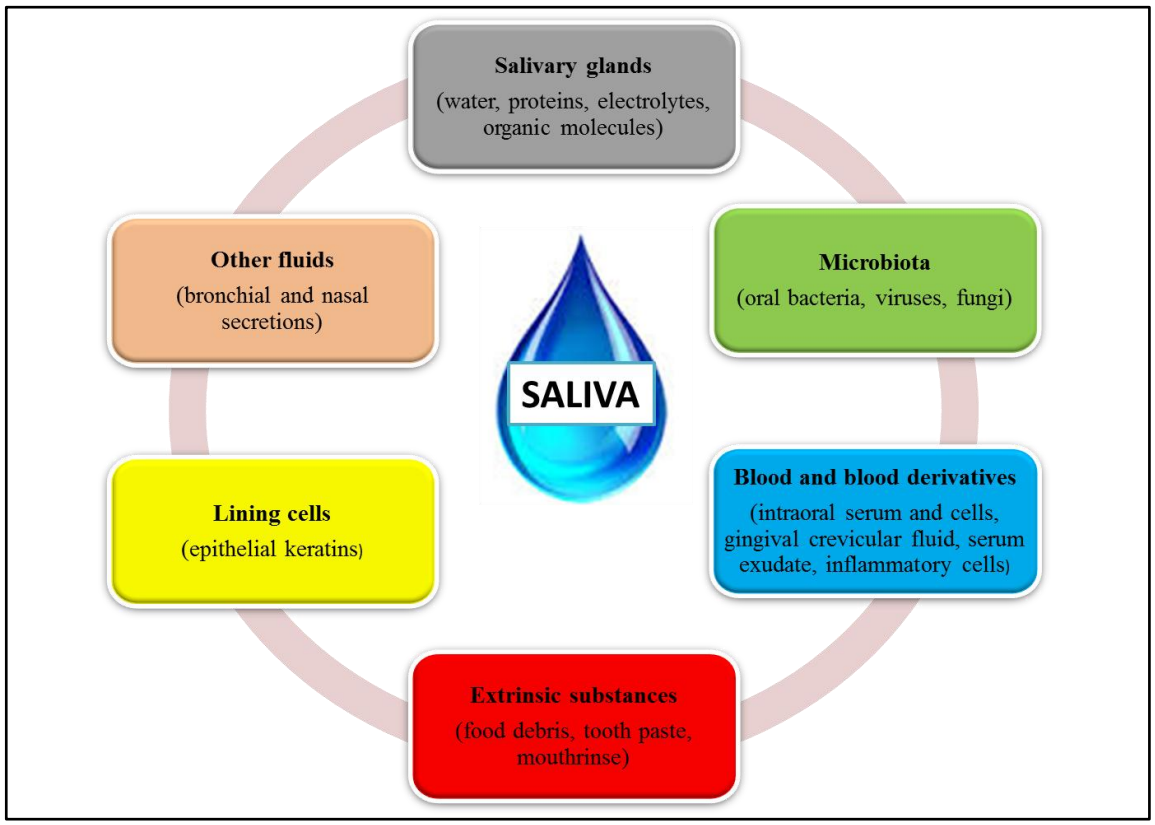



**Figure 1.11** Components of whole saliva. Adapted from (Kaufman and Lamster, 2000).

### 1.4.2.3 Function of saliva

The complexity of saliva reflects the multiplicity of its functions. In addition to its lubricating mechanism, it provides extensive protection from chemical, mechanical and thermal irritation to both soft and hard oral tissues; acts as an ion reservoir to enhance tooth remineralisation; reduces tooth demineralization by way of a buffering mechanism that neutralizes the pH of plaque; stimulates antimicrobial activity to help control the oral micro flora with a presence of antibodies, molecules and various enzymes; aids digestive action through its alpha amylase enzyme in order to breakdown starchy foods; facilitates food tasting through dissolving activities that allow for interaction between food particles and taste buds; produces salivary proteins to assist in the formation of a pellicle and protein matrix for the plaque, forming an acquired enamel protection barrier; and finally, saliva facilitates air flow, speech and swallowing (Welton, 2012) (Figure 1.12).

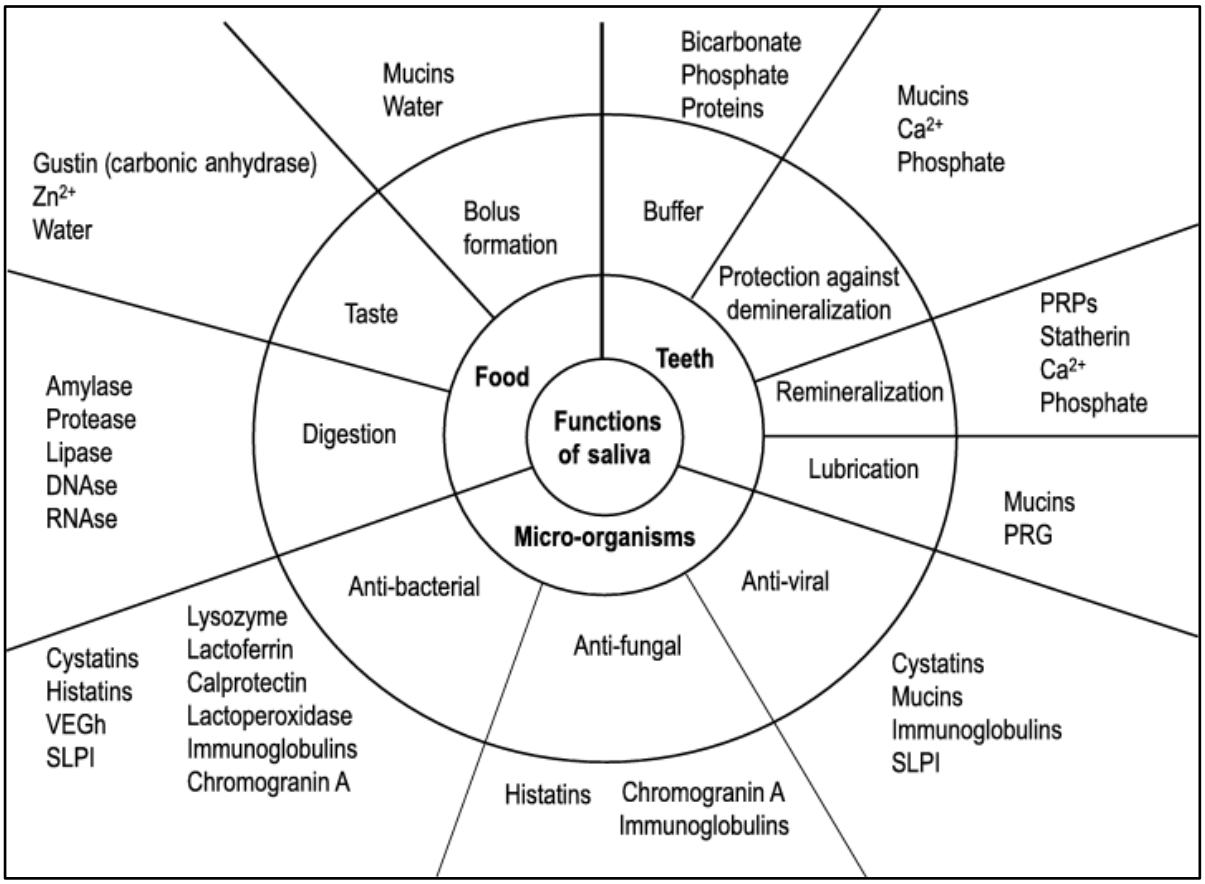



**Figure 1.12** Overview of the main functions of saliva in relation to its organic and inorganic constituents. Acknowledgments to (Amerongen and Veerman, 2002).

### 1.4.2.4 Salivary flow

Daily salivary flow rate is approximately 0.5-0.6 litres/day, with a value below 0.1 ml/min being considered hyposalivation and a value above 0.4 ml/min being considered hypersalivation (Flink et al., 2005). This flow rate differs for stimulated and unstimulated saliva (Dowes, 2012).

The flow rate for unstimulated saliva normally averages 0.3-0.4 ml/min (Flink et al., 2005), with the parotid generating around 25% of this, the submandibular forming 60%, 7-8% coming from sublingual glands, and a further 7-8% accumulating from the minor salivary glands (De Almeida et al., 2008). For stimulated saliva, an average flow rate of 1.5-2.0 ml/min comprises 50% from the parotid glands, 35% from the submandibular glands, 7-8% from the sublingual glands, and the same percentage from the minor salivary glands (Dowes, 2012).

Salivary flow rate and its composition can be influenced by a wide range of factors, such as systemic medical conditions (depression, allergies and hypertension), individual hydration, body posture, lighting, smoking, the circadian and circannual cycles, thinking about food, visual stimulation, and medication. Stimulated salivary flow rate is also affected by the regular stimulation of the salivary flow, size of salivary glands and body weight (De Almeida et al., 2008). Although many elderly people suffer from a dry mouth, it has been reported that this does not result from low salivary flow but is more likely to be due to the effects of medication and some systemic illnesses. A higher saliva flow rate is more effective for the reduction of demineralization and the promotion of re-mineralization of the teeth, leading to an increased chance of calculus formation (Dowes, 2012).

### 1.4.2.5 Saliva as a diagnostic tool

Saliva is seen as an ideal diagnostic bio-medium and provides an excellent alternative to other body fluids for the purposes of investigation. It is easily collected, stored and transported, while also being safe to handle in comparison to other biological media. Furthermore, sample collection can be repeated frequently throughout the day without the need for skilful personnel, therefore it is anticipated that salivary diagnosis will be particularly useful in cases where repeated samples of body fluid are required and the drawing of blood impractical and/or unethical (Pfaffe et al., 2011).

It is a well-known fact that saliva contains a wide range of proteins, peptides, electrolytes, nucleic acids and hormones. These originate from various sources, with its biochemical and immunological components, for example, coming from a salivary gland itself or through the passive or active diffusion of blood (Aps and Martens, 2005, Chiappin et al., 2007).

Accordingly, saliva has been discussed not only as an important biological material for diagnostic tests, but also for the explanation of the pathogenesis of numerous systemic diseases, such as leukemia, Sjogren's syndrome, AIDS, systemic lupus erythematosus, and diabetes mellitus. As demonstrated by the increasing use of this biological fluid for diagnostic purposes in oral pathologies, salivary analysis may aid in the identification of many diseases, especially those affecting the oral cavity (Mandel, 1990, Streckfus and Bigler, 2002). Non-invasive salivary testing may prove an effective modality for the diagnosis and prognosis of oral cancer, and the monitoring of a patient's post-therapy status (Nagler et al., 2003).

The pathogenesis factor and salivary components appear to be closely associated, as confirmed by studies into salivary biochemical and antioxidants systems in several pathological conditions (Nagler et al., 2003; Aizenbud et al., 2008). Various enzymes can be found among the important components of saliva that are proposed as disease markers. The damaged cells of periodontal tissue increasingly release intracellular enzymes into the GCF and saliva. Further studies suggest that saliva analysis may provide a cost-effective approach for the assessment of periodontal diseases in populations (Kaufman and Lamster, 2000). However, a range of studies present conflicting results regarding various individual salivary agents and the link that they might have with oral health, in particular for dental caries (Numabe et al., 2004).

It was also shown that different salivary components can be used for the diagnosis of oral conditions and monitoring the course of treatment, such as enzymes and immunoglobulins, hormones of host origin, bacteria and bacterial products, ions, and volatile compounds. However, to date no one chemical agent is more important than any other. A significant number of the defence factors show additive or synergistic interactions against oral pathogens (Mandel, 1990, Kinney et al., 2007). A lack of saliva or saliva deficiency results in negative implications for both oral health and general body health (Pinka et al., 2009).

### 1.4.2.6 Saliva collection methods

Saliva can be collected and measured as unstimulated whole saliva or stimulated saliva. Unstimulated whole saliva covers, moisturizes and lubricates oral tissues and provides the normal baseline present in oral cavity during a 24-hour period. It often correlates with systemic clinical conditions more accurately than stimulated saliva does due to the fact that the materials used to stimulate flow may change the composition of saliva (Munro et al., 2006).

Whole-mouth resting saliva is collected using one of the following methods: draining, drooling, spitting, swabbing or suction (Navazesh, 1993). More recently, the alternative technique of using filter paper placed in the sublingual pocket for unstimulated saliva collection has shown numerous potential advantages over other known procedures (Neu et al., 2007).

Mechanical, olfactory, gustatory or pharmacological stimuli are used to stimulate saliva, which is collected either by having the patient chew on a piece of paraffin and/or by applying 0.1– 0.2 ml/l (approximately 1 drop) of citric acid to the tongue (Navazesh, 1993). Saliva can also be probed from specific glands via cannulation of the glandular ducts, or through the use of certain collecting devices at the site where the glandular ducts emerge (Edgar et al., 2004).

### 1.4.2.7 Salivary changes with periodontal diseases

Periodontal disease is the most noteworthy condition that can be diagnosed via saliva analysis since various enzymes, cytokines and biomarkers of bone turnover are present in saliva in cases of periodontitis in comparison to saliva pertaining to healthy periodontal status (Teles et al., 2009, Kinney et al., 2011). In addition, while both locally and systemically derived biomarkers of host origin are contained in saliva, further microbial markers for periodontal disease are also present (Kaufman and Lamster, 2000, Koh and Koh, 2007). Nevertheless, it must be noted that the detection of the active disease site is difficult in saliva analysis and, furthermore, such an analysis is affected by factors such as salivary flow rate, medications and smoking (Gursoy et al., 2010).

Several studies have been conducted to investigate certain biomarkers in saliva for the early detection and management of periodontitis, the earliest biomarker investigated was IL-1β. Many studies concluded significantly higher levels of IL-1β in the periodontitis

group comparing to the healthy ones (Tobón-Arroyave et al., 2008, Gursoy et al., 2009, Ebersole et al., 2013), others found same level of IL-1β with and without periodontitis (Christodoulides et al., 2007, Teles et al., 2009). Alternative studies showed same levels of salivary TNF-α in patients with periodontitis and healthy subjects (Gursoy et al., 2009, Mirrielees et al., 2010). In addition to the cytokines, significantly higher levels of adipokines such as visfatin and chemerin have been observed in saliva of patients with periodontitis comparing to periodontal health subjects (Tabari et al., 2014, Özcan et al., 2015). Salivary RANK and OPG were measured in patients with periodontitis, some studies showed same levels of OPG in diseased and healthy groups (Miller et al., 2006, Costa et al., 2010), others mentioned that OPG levels declined following periodontal treatment (Kinney et al., 2011, Sexton et al., 2011). Another study mentioned that the salivary levels of RANKL were the same in periodontitis and healthy groups (Frodge et al., 2008), however other studies reported that salivary RANKL was significantly higher in periodontitis group in comparison to the healthy one (Tobón‐Arroyave et al., 2012, Tabari et al., 2013). Further research demonstrated significantly higher levels of growth factors in saliva with periodontitis (Wilczynska-Borawska et al., 2006, Rudrakshi et al., 2011, Wilczyńska-Borawska et al., 2012).

### 1.4.2..8 Salivary changes with orthodontic treatment

OTM takes place through bone remodelling that is used primarily to enable bones to respond and adapt to mechanical stress during orthodontic treatment (Van Nieuw Amerongen et al., 2004, Ariffin et al., 2010). Due to such treatment, lactate dehydrogenase levels increase in saliva during bone remodelling. For the time being, clinical and radiographic follow-up examinations comprise the main method for patient evaluation. However, saliva analysis may provide a foundation for phase-specific screening in OTM (Flórez-Moreno et al., 2013b).

During orthodontic treatment, salivary flow rates have been shown to increase 1-3 months after the delivery of fixed appliances in comparison to the baseline measured both before starting treatment and at the control levels (Li et al., 2009). It has also been shown that 90-180 days after the completion of orthodontic treatment, the salivary flow rate moved closer to the baseline and control levels. Research into the cariogenic bacterial counts in whole saliva have found that 3 months after the delivery of the fixed appliance,

bacterial counts increased significantly before returns to baseline levels (Peros et al., 2011). Other studies illustrate associations between specific genotypes and susceptibility to root resorption (Al-Qawasmi et al., 2003), speed of OTM (Iwasaki et al., 2009), and primary eruption failure (Frazier-Bowers et al., 2010). More recently, saliva, collected in sufficient volume, has been employed to identify genotyping instead of blood and buccal swabs (Pulford et al., 2013).

The biocompatibility of orthodontic appliances was investigated by analysing the metal ion released from the materials of the appliances into saliva; the results showed that most of ions were released during the initial stage of the treatment (Mikulewicz and Chojnacka, 2010). Salivary alpha-amylase activity was measured as a possible indicator for pain during orthodontic treatment, the results showed no correlation between pain intensity during orthodontic treatment and salivary levels of alpha-amylase (Campos et al., 2011). An alternative study examined the salivary concentration of bone remodelling biomarkers such as deoxypyridinoline and bone-specific alkaline phosphatase during orthodontic treatment, the outcomes of this prospective follow up study indicated that although both of these biomarkers may indicate increased bone remodelling. It appears that only deoxypyridinoline increased in the earlier phases of OTM, whereas bone-specific alkaline phosphatase might act as an indicator of bone formation at the end of tooth movement (Flórez-Moreno et al., 2013b).

A different research paper linked the salivary levels of soluble RANKL, OPG and RANKL/OPG ratio to the phases of orthodontic treatment and reported that their levels might assist clinically in the monitoring of orthodontic treatment (Flórez-Moreno et al., 2013a). IL-1β, TNF-α, malondialdehyde, nitric oxide, and 8-hydroxydeoxyguanosine were investigated in saliva of patients with fixed orthodontic appliances before treatment, at 1st month and at 6th month of treatment. The findings showed that there were no significant changes in the levels of these analytes at any time point indicating that changes with OTM do not exceed the physiological limits of these analytes in saliva (Atuğ Özcan et al., 2014). The level of inflammation during orthodontic treatment was assessed by measuring MPO activity in saliva and GCF at baseline, 2 hours, 7 days and 14 days after the activation of orthodontic appliances. MPO activity increased until day seven with the highest activity was at 2 hours and values were reduced to baseline level at day 14 in the both GCF and saliva samples (Navarro-Palacios et al., 2014).

### 1.4.2.9 Salivary changes with obesity

In recent years, it has been suggested that the determination of salivary adipokines may contribute to the elucidation of the physiology and role of adipokines not only in the development of obesity and insulin resistance, but also in inflammation, lack of energy balance or the stress response. For instance, a study mentioned a positive correlation between the salivary adiponectin concentrations with its circulating concentration of healthy individuals. However, the levels of salivary visfatin did not correlate with the concentration in serum (Mamali et al., 2012). Some reports indicated a significant increase in the concentration of salivary CRP (a sensitive marker of systemic inflammation) in obese children than in children of normal weight (Naidoo et al., 2012, Goodson et al., 2014). Similarly, correlation was also observed between serum and salivary uric acid, systolic and diastolic blood pressure, waist circumference, BMI, blood glucose, triglycerides, high-density lipoprotein and the number of cardiometabolic risk factors (Soukup et al., 2012). A reduced level of antioxidants was also observed in obese individuals not only in serum but also in saliva (Bhardwaj et al., 2008).

Three times more bacterial cells were observed in the subgingival biofilm with six more bacterial species in the saliva of obese adolescents in comparison to normal weight (Zeigler et al., 2012). In the same field, another study evaluated the salivary conditions of morbidly obese patients prior and 6 months after bariatric surgery, finding that obese patients undergoing bariatric surgery presented with a higher microbiological level of mutans streptococci in saliva after 6 months (Hashizume et al., 2015).

Some salivary parameters have been examined in stimulated saliva in relation to BMI such as pH, flow rate, buffer capacity, proteins concentration, phosphate, calcium, sialic acid and peroxidase activity. The authors suggested that overweight and obesity lead to increase in concentrations of sialic acid, protein, and reduce phosphorus as well as peroxidase activity which may promote dental caries (Pannunzio et al., 2010).

In more elaborated investigations salivary flow rate has also been measured in relation to obesity. A low salivary flow rate was observed with childhood obesity (Modéer et al., 2010), with BMI>25 in adults less than 50 years old (Flink et al., 2008) and in morbidly obese (BMI>40) (Knaś et al., 2015) which further strengthens the negative influence of obesity on oral health. Conversely, other study reported comparable salivary pattern in obese and normal-weight individuals (Epstein et al., 1996).

## 1.5 Thesis aims and objectives

Obesity is recognised as a low-grade systemic inflammatory disease, which modifies the host immune response, resulting in an increased risk of infections and a systemic pro-inflammatory state. In oral health, there is a close relationship between obesity and periodontitis due to the exacerbated pro-inflammatory changes that take place in periodontal disease. Since orthodontic treatment mainly depends on tissue and bone remodelling within the periodontium, obesity can potentially affect orthodontic therapy; in particular, through pro-inflammatory changes within the PDL space that could have an impact on tissue remodelling within the PDL.

During OTM teeth are exposed to mechanical stimuli that alter the vascularity of periodontal tissue and blood flow, leading to local synthesis and release of different biological molecules (biomarkers). The sequence of biological mechanisms that control the shift from stimulus (orthodontic force) to reaction (tooth movement) can be monitored and evaluated in relation to higher or lower levels of relevant biomarkers. Therefore, biochemical analysis is a useful and promising method to monitor the changes during a certain period and to investigate the response of dental and paradental tissues to OTM.

There is some evidence of variation in the levels of biomarkers in obese and normal-weight individuals with periodontal disease; however, data relating to biomarkers within this population during OTM is sparse. Although some retrospective studies have found that obesity can affect orthodontic therapy potentially through its effect on bone metabolism, growth and development, currently no study exists to investigate OTM in obese patients, from either a clinical or biochemical perspective.

In humans, different biological samples have been used to monitor periodontal disease progression such as saliva, GCF, plaque biofilm and blood. Biomarker analyses during initial OTM needs to be investigated in GCF samples, as it is non-invasive and closely positioned to the periodontium where tissue and bone remodelling takes place. However, the components of GCF consequently pass to saliva, which is easier, less invasive and less expensive to collect and analyse. We are interested in studying OTM in association with obesity, and therefore serum samples also need to be analysed. The rationale behind this is that adipokines, are mainly produced by adipocytes and secreted in blood as the first transporter. Therefore in this study, three different samples, namely UWMS, GCF and serum, were used to assess the levels of the selected biomarkers.

The main aim of this investigation was to examine the influence of obesity on OTM in adolescent subjects at both the clinical and biochemical levels.

In order to achieve this aim the following objectives were undertaken:

1- Measure the effects of obesity on biochemical changes in UWMS, GCF and serum of humans with and without orthodontic treatment;
2- Measure rate of OTM and time taken to achieve completion of tooth alignment using fixed orthodontic appliances in a cohort of normal weight and obese patients;
3- Biochemical analysis of inflammation, tissue remodelling, bone metabolism and obesity-related biomarkers in different bio-fluids (UWMS, GCF and serum) of normal weight and obese subjects to assess which fluid is more representative for biological changes seen during OTM; and
4- Measure pain experience and analgesic intake during initial alignment with fixed orthodontic appliances in normal weight and obese patients.

# Chapter 2 Participants and methods

## 2.1 Ethical approval

This study was approved by the Human Research Ethics Committee of King's College London (BDM/14/15-8, November 2014), and the United Kingdom National Research Ethics Service, NRES Committee foundation (14/LO/0769, October 2014). Informed written consent was obtained from all participants.

## 2.2 Body mass index measurement

In order to classify the participants into normal weight and obese, BMI was measured as follow:

### 2.2.1 Body mass index for adults

Height and weight of participants were measured to calculate BMI as weight in kilogram divided by square height in meter ($kg/m^2$). The body weight of subjects was measured to the nearest 0.1 kilogram (kg) using a calibrated scale and height was measured to the nearest centimetre (cm) using a wall-mounted ruler. According to WHO, BMI classified as <18.5 underweight, 18.5-24.9 healthy (normal weight), 25-29.9 overweight, 30-39.9 obese, ≥40 morbidly obese. Participants who were classified as underweight and overweight were excluded from the study.

### 2.2.2 Body mass index for growing subjects

Height and weight of participants were measured to calculate BMI ($kg/m^2$) similar to adults by measuring the body weight to the nearest 0.1 kilogram (kg), using a calibrated scale and the height to the nearest centimetre (cm) using a wall-mounted ruler. The Royal College of Paediatrics and Child Health (RCPCH) UK WHO growth charts (2013) were used to calculate and classify BMI centile in relation to age and sex (Appendices 1 and 2). Accordingly, subjects were classified as underweight (BMI centile <2), normal weight (BMI centile 2-91), overweight (BMI centile 91-98) and obese or morbidly obese (BMI

centile >98). Subjects who were classified as underweight and overweight were excluded from the study.

## 2.3 Periodontal health

In order to adjust the possible interactions of bacterial plaque and gingival inflammation on the concentrations of biomarkers, the periodontal health was measured using established and validated gingival (Löe and Silness, 1963) and plaque (Silness and Löe, 1964) indices, which are characterised as follow:

### 2.3.1 Dental plaque

Dental plaque thickness adjacent to gingival margin was assessed following the criteria of (Silness and Löe, 1964), while Ramfjörd index teeth were examined to represent the whole dentition (Ramfjord, 1959). In this study, four surfaces of each tooth were examined and these included mesial, buccal, distal and lingual surfaces according to the criteria for plaque index as:

0: No plaque

1: A film of plaque adheres to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using probe on the tooth surface.

2: Moderate accumulation of soft despites within the gingival pocket, or the tooth and the gingival margin, which can be seen with the naked eye.

3: An abundance of soft matter within the gingival pocket and/or the tooth and gingival margin.

### 2.3.2 Gingival health

For the diagnosis and assessment of gingival health a validated gingival index has been described (Löe and Silness, 1963). The four surfaces of Ramfjörd index teeth were examined to represent the whole mouth as that done for dental plaque measurement (Ramfjord, 1959). The criteria for gingival index as:

0: Absence of inflammation / normal gingiva.

1: Mild inflammation: slight change in colour, slight oedema, no bleeding on probing.

2: Moderate inflammation: moderate glazing, redness, oedema, and hyperatrophy, bleeding on probing.

3: Severe inflammation: marked redness and hyperatrophic ulceration, tendency to spontaneous bleeding.

## 2.4 Participants and sample collection

Three groups of participants were recruited as follows:

1) Normal weight (BMI = 18.5-25) and obese (BMI ≥ 30) adult participants without orthodontic treatment (control cohort), with a minimum age of 18 years and a maximum age of 45 years were purposively recruited with a matched age and gender from students and researchers from King's College London Dental Institute (Guy's Hospital). They have clinically acceptable gingival health, no artificial prosthesis or orthodontic appliance or retainers, no history of illness or the taking of medication within the previous 3 months, were not pregnant and non- smokers;
2) Normal weight (BMI = 18.5-25) and obese (BMI ≥ 30) adult patients undergoing fixed appliance orthodontic treatment with 0.019 x 0.025-inch stainless steel rectangular archwires in the upper and lower arches with a minimum age of 18 years and a maximum age of 45 years were purposively recruited with a matched age and gender from patients attending for routine fixed-appliance treatment at King's College London Dental Institute (Guy's Hospital). They have no history of illness or the taking of medication within the previous 3 months, were not pregnant and non- smokers; and
3) Normal weight (BMI centile= 2-91) and obese (BMI centile ≥ 98) adolescent patients with a minimum age of 12 years and a maximum age of 18 years were purposively recruited with a matched age and gender from patients attending for routine fixed-appliance treatment at King's College London Dental Institute (Guy's Hospital). They have permanent dentition; mandibular arch incisor irregularity index of 4-12 mm, need fixed orthodontic appliance treatment with or without teeth extraction, no medical contraindications or regular medication (including antibiotic-therapy in previous 3 months); and non-smokers.

From each individual the following samples were collected during the day between the hours 9:30 am to 3:30 pm:

1) UWMS was collected by asking the subject to passively drool in a pre-weighed sterile plastic tube for 5 minutes. The sample was put in an icebox containing ice after collection and carried to the laboratory where it processed and stored. The processing procedure of the collected saliva started with weighing the tubes with the sample again (to get the net saliva volume), vortexing them for 20-30 seconds and centrifuging the samples at 9200 g for 5 minutes. The processed saliva was aliquoted into small Eppendorf Tubes® (1000 µl capacity), labelled and stored at – 80 °C in freezers until the time for analysis.
2) Samples of GCF were collected using Periostrip (OraFlow Inc. New York, USA), from the distal side of the lower incisors and canines because the rate of alignment of these teeth was measured using Little's Irregularity Index. Plaque was removed gently by a strip of cotton held in tweezers, the teeth were dried and the surrounding area isolated with cotton-wool rolls. Periostrip was placed 1 mm sub-gingivally into the gingival crevice for 30 seconds (Figure 2.1). If there was any contamination of the strip with saliva or blood it was discarded. Each strip was placed in an individual Eppendorf Tubes® (500 µl capacity, Alpha Laboratories, UK) and immediately transferred to an icebox containing ice. Then the collected samples were carried to the laboratory where they were processed and stored. The processing procedure of GCF started with measuring the volume of the collected GCF by placing the strip between the upper and lower counterparts of the Periotron 8000 electronic micro-moisture meter (OraFlow Inc. New York, USA) (Figure 2.2), with readings converted to an actual volume by reference to the standard curve and measured the diluting factors of the collected GCF. Proteins in the Periopaper were eluted with 20 µl phosphate buffered saline (PBS) for each strip, centrifuged at 9200 g for 5 minutes while the handle of the strip was secured under the lid. Then the eluted GCF was aspirated from all Eppendorf tubes, pooled, aliquoted into small Eppendorf Tubes® (500 µl capacity, Alpha Laboratories, UK), labelled and stored at – 80 °C in freezers until the time for analysis.
3) Peripheral blood was collected by finger pinprick using a disposable sterile needle (ACCU-CHEK, Safe-pro, Germany). A few droplets of blood were collected with a 3-4 sterile Sialostrips (OraFlow Inc. New York, USA). Each strip placed in individual Eppendorf Tube® (500 µl capacity, Alpha Laboratories, UK) and transferred to icebox containing ice immediately. The samples were carried to the laboratory, processed and the volume and diluting factor were measured in the same way mentioned for GCF, but

each strip was eluted using 100 µl PBS and centrifuged at 5900 g for 5 minutes to separate the blood.Then, the supernatant serum was aspirated from all Eppendorf tubes, pooled, aliquoted into small Eppendorf Tubes® (500 µl capacity, Alpha Laboratories, UK), labelled and stored at – 80 °C in freezers until the time for analysis.

**Figure 2.1** Gingival crevicular fluid collections.

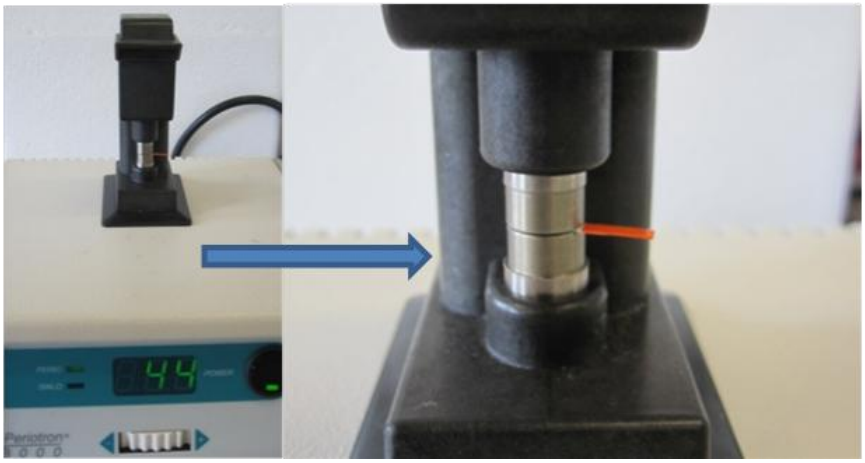


**Figure 2.2** Periotron 8000 used for the measurement of GCF and blood volume. Periotron was cleaned and dried after each sample.

## 2.5 Stimulated parotid saliva collection

Stimulated parotid saliva was collected using a parotid saliva collector (lashley cup, King's Medical Science, UK), Tygon tubing (10.2 cm for the collecting tube from the inner circle of the parotid saliva collector, and 30.5 - 40.6 cm to connect the parotid collector to syringe) and 3 ml Luer-lock syringe attached to a blunt cannula.

The orifice of the parotid duct is located on the buccal mucosa opposite the upper second molar tooth. The area was dried and the parotid collector was placed on the mucosa so that the inner ring surrounded the duct orifice. The collector is held on the mucosa by suction from the outer ring, created by pulling back on the syringe and allowing the pressure to come to equilibrium. A medium binder clip is attached to the Tygon tubing going from the collector to the syringe to "lock-in" the air in the tubing.

The suction created should be sufficient enough to hold the cup in its place without occluding the inner chamber of the parotid collector with tissue (not too much suction). Stimulation is performed by applying 2% citric acid solution to the posterior lateral surfaces of the tongue, bilaterally, using 2 drops every 30 seconds to stimulate secretion. Saliva from the parotid gland then flows passively into the inner ring and through the attached tubing. The flowing saliva then collected into an ice-cooled pre-weighed and pre-labelled container.

## 2.6 Flow rate

As the salivary and GCF flow rate can be influenced by the inflammation and mechanical stimulation, the UWMS and GCF flow rates were measured to adjust the possible interaction with the concentration of biomarkers.

### 2.6.1 UWMS flow rate

The measurement of UWMS flow rate was performed by weighing the tubes with the sample and subscribed from the original weight of tubes before sample collection to get the net saliva weight in gram. Then the net weight divided by the duration of sample collection (5 minutes) to measure the salivary flow rate per minute (ml/min).

### 2.6.2 GCF flow rate

GCF flow rate calculation was started by measuring the volume of the collected GCF from all strips using the Periotron, with readings converted to an actual volume by reference to the standard curve.

Since GCF was collected for 30 seconds, the total volume multiplied by 2 to get the volume per minute, and then divided by the number of the selected teeth in order to calculate the GCF flow rate for a tooth per minute.

## 2.7 Total protein concentration

Total protein concentration of sample was measured in UWMS, eluted GCF and eluted serum using the NanoDrop (software V3.5.2, Thermo Fisher Scientific, UK). The Nanodrop was calibrated with 2 µl of nuclease free water. Then 2 µl from each sample was applied to the Nanodrop and protein concentration was measured at 280 nm with setting 1 Abs=1 mg/ml. Triplicate measurements were made for each sample and the mean average was calculated.

## 2.8 Sample preparation and electrophoresis

All samples were prepared under reducing conditions with 5% NuPage lithium dodecyl sulphate sample buffer (LDS, Invitrogen, Thermo Fisher Scientific, UK), 10% 0.5 M dithiothreitol (DTT) and heated for 3 minutes at 100 °C. The samples were either stored at -20 °C or used for analysis immediately.

Samples were analysed for protein content using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). 10 µl of samples and 5 µl of SeeBlue Plus Pre-stained marker (Invitrogen, Thermo Fisher Scientific, UK) were loaded into pre-cast NuPage Novex, 4-12% Bis Tris gel (Novex, Life Technologies, UK).

These gels were set up in an Xcell vertical electrophoresis unit (Invitrogen, Thermo Fisher Scientific, UK) with 25 ml NuPage MES SDS running buffer (Invitrogen, Thermo Fisher Scientific, UK) and 475 ml distilled water and electrophoresed for 35 minutes at 125 mA and 200 volts constant.

In order to detect proteins in the loaded samples, gels were stained once using Silver stain, Coomassie Brilliant Blue stain and Periodate Schiff stain (PAS).

## 2.9 Immuno-detection of proteins

Electrophoretic transfer of protein (Western blot) from SDS-PAGE gels to nitrocellulose membranes (Whatman Protan; Dassel, Germany) was carried out for the detection of specific proteins.

For Western blotting, a nitrocellulose membrane was placed on top of the SDS-PAGE gel and sandwiched between filter paper and sponge saturated and packed with

NuPage Transfer Buffer of 25 ml NuPage transfer buffer (Invitrogen, Thermo Fisher Scientific, UK), 50 ml methanol and 425 ml double distilled water.

The sandwich was secured in an Xcell vertical electrophoresis unit (Invitrogen, Thermo Fisher Scientific, UK) and filled with the remaining transfer buffer. Transfer was carried out at 30 volts constant and 150 mA for 60 minutes. At the end of the transfer, the gel was discarded and the membrane placed (contact side up) in a clean disposable tray for protein detection.

For immunoblotting, the membrane was blocked with Tris Buffered Saline with Tween (TBST) solution for 1 hour with gentile agitation on a rocker. The primary antibody, with specific dilution, was added to the membrane in the TBST for 1 hour with gentle agitation. The membrane was washed with TBST 3 times, 5 minutes each, then incubated with a secondary antibody diluted 1:2000 with TBST for 1 hour with gentle agitation. The solution discarded and the membrane washed as previously described. Antibody signal was detected using chemiluminescent substrates (Immun-Star Western C, Bio-Rad Laboratories, Inc, UK). The detection signal was visualized using the chemi Doc $^{TM}$ Imaging system (BioRad) laboratories ltd, Herts, UK.

## 2.10 Zymography

For more specific detection of MMPs, samples were also analysed for gelatinase using 10% zymogram gelatin gels electrophoresis of Novex, Life Technologies, UK. Using X-cell vertical electrophoresis unit with 1X Tris-Glycine SDS running buffer (Novex, Life Technologies, UK) (100 ml 10X Tris-Glycine SDS running buffer to 900 ml deionized water). 5 µl of samples and 5 µl of Tris-Glycine SDS sample buffer (2X) (Novex, Life Technologies, UK) was loaded per well and electrophoresed for 90 minutes at 30 - 40 mA and 125 volts constant.

Samples were denatured in SDS buffer under non-reducing conditions and without heating, run on a Novex®Zymogram gel (Novex, Life Technologies, UK) using Tris-Glycine SDS Running Buffer (2X). After electrophoresis, the enzyme was re-natured by incubating the gel in (1X) zymogram re-naturing buffer (Novex, Life Technologies, UK), for 30 minutes. The gels were then equilibrated in (1X) zymogram developing buffer (Novex, Life Technologies, UK) for another 30 minutes.

The equilibrated gel was incubated in fresh zymogram developing buffer overnight for maximum sensitivity and then stained with Coomassie blue stain, and de-stained. Regions of gelatinase activity appeared as clear bands against a dark blue background where the gelatinase has digested the substrate.

## 2.11 Mass spectrometry identification of proteins (proteomics)

In order to identify the positively detected proteins in the gel, mass spectrometry was performed.

Under heating and reducing conditions, purified adiponectin standard (Recombinant human adiponectin (NSO-derived)/Acrop30, CF, R&D systems-Bio-Techne, UK), UWMS, eluted GCF and eluted serum were loaded in the same gel twice. After electrophoresis, proteins in one half of gel were transferred to nitrocellulose membrane by Western blot for detection of adiponectin by immunoblotting. Proteins of interest were identified by immunoblotting then cut from the second half and sent for proteomic analysis at the Centre of Excellence for Mass Spectrometry, King's College London, to detect the proteins by a specialist technician.

The desired sections were then reduced, alkylated and subsequently digested with trypsin (Promega, Madison,WI, USA) at 37 $^{o}$C for 2 hours then overnight at room temperature. The reaction was stopped by formic acid. Digests (peptides) were extracted, desalted and cleaned up then separated, eluted and ionised using Reverse phase- High-Performance Liquid Chromatography RP-HPLC. Ionised molecules (precursors and fragmented) were measured (scanned) using MS detector (scan). All LC-MS/MS data were searched using the MASCOT database search engine (Matrix Science, London, UK) against human Swis-sprot protein database (uniprot sprot 130220 database) to obtain peptide and protein identifications. MASCOT results were imported into Scaffold version 4 (Proteome Software, Portland, OR, USA) and viewed.

## 2.12 Enzyme-linked-immuno-sorbant assay

In order to measure the concentration of specific protein (adiponectin), enzyme-linked-immuno-sorbant assay (ELISA) was performed. The wells of 96-well microtitre plate

(Thermo Scientific, UK) were coated overnight at 4 $^{o}$C with 100 µl of unconjugated capture antibody (mouse monoclonal IgG purified, R&D systems-Bio-Techne, UK) diluted to 1:2000 in pH 9.6 sodium carbonate buffer (0.1 M). The wells were washed 3 times with phosphate buffer saline and 0.1% Tween 20 (PBS-T). 200 µl of the standard (1 µg/ml of recombinant human adiponectin (NSO-derived)/Acrop30, CF, R&D systems-Bio-Techne, UK) was added in duplicates of 2-fold serial dilutions. 100 µl of UWMS, GCF and the serum of 20 volunteers was added in duplicates.

After 1 hour incubation at 37 $^{o}$C, the plate was washed 3 times with PBS-T. A 1:500 dilution of the detecting antibody (biotinylated mouse monoclonal IgG, R&D systems-Bio-Techne, UK) was incubated on the plate for 1 hour at 37 $^{o}$C. Following a third wash with PBS-T, 100 µl of horseradish peroxidase streptavidin (Vector laboratories, USA) was added for all wells and incubated at 37 $^{o}$C for 1 hour followed by 3 times washing with PBS-T. 100 µl of substrate solution contained 250 µl of 3, 3', 5, 5' tetramethylbenzidene stock solution (3 mg/ml in DMSO) and 3 µl hydrogen peroxide in 20 mls of sodium acetate buffer (100 mM, pH 5.5) was added. The reaction was stopped after colour changing by the addition of 50 µl of 2 M sulphuric acid and the absorbance at 450 nm read in a microplate reader (Bio-Rad, iMark, UK). The data were exported to an Excel spreadsheet and multiplied by a diluting factor to get the final values for statistical analysis.

## 2.13 Detection and quantification of biomarkers by magnetic Luminex multiplex assay

In order to measure the concentrations of many proteins in the same small volume of sample, especially GCF because of its limited volume, magnetic Luminex screening assays (R&D systems-Bio-Techne, Abingdon, UK) were carried out for 3 types of sample according to the manufacturer's instructions. Specific antibodies are pre-coated onto colour-coded magnetic microparticles. The microparticles, standards and samples were all pipetted into wells and the immobilized antibodies bind the protein of interest. The plate was washed to remove any unbound substance and a biotinylated antibody cocktail specific to the protein of interest was added. After another wash, streptavidin-phycoerythrin conjugate (SA-PE) was added to each well, which bound to the biotinylated antibody. Measurements were made using Bio-Plex Manager $^{TM}$ 6.1 software of a Luminex machine

(Bio-Plex 200 system with HTF, Bio-Rad Laboratories Ltd, UK). The magnitude of the phycoerythrin-derived signal, which is proportional to the amount of the analyte bound, was analyzed using Bioplex Manager 6.0. Table 2.1 demonstrates the upper and lower detection values for the selected biomarkers. The data were exported to an Excel spreadsheet and multiplied by diluting factor to get the final values for statistical analysis (Figure 2.3).

**Table 2.1** Limits of detection for biomarker Luminex analysis.

| Biomarker * | Lower limit of detection (pg/ml) | Upper limit of detection (pg/ml) | Coefficient of variation † (%) |
|---|---|---|---|
| Adiponectin | 193.74 | 432390.10 | 2.4 |
| Leptin | 55.15 | 111251.22 | 3.0 |
| Resistin | 4.83 | 9709.28 | 1.8 |
| MMP8 | 25.45 | 53522.68 | 2.3 |
| MMP9 | 20.03 | 41870.79 | 2.8 |
| TIMP1 | 7.91 | 17479.14 | 2.1 |
| MPO | 16.31 | 34627.04 | 2.8 |
| CRP | 17.29 | 38785.30 | 2.4 |
| RANKL | 5.14 | 10509.81 | 2.3 |

* all manufactured by R&D Systems. MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; MPO, myeloperoxidase; CRP, C reactive protein; RANKL, receptor activator of nuclear factor kappa-B ligand

† mean coefficient of variation was calculated by measuring the same standard on 10 plates and dividing the standard deviation by the average value (*100)

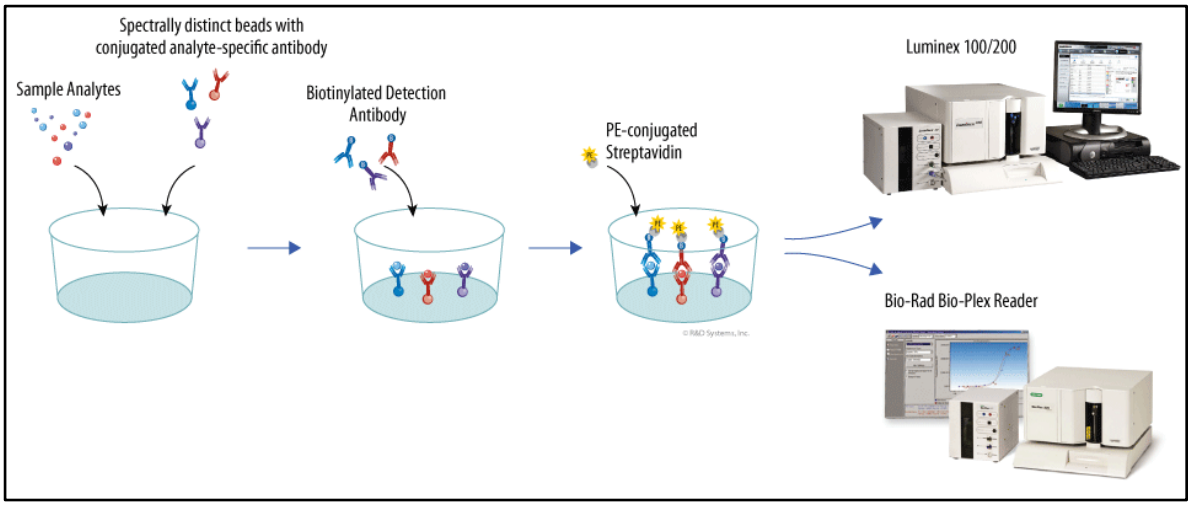



**Figure 2.3** Principle of Luminex multiplex assay. Adapted from (Fuerstenberg et al., 2014).

# Chapter 3 Biomarker detection in different bio-fluids

## 3.1 Introduction

Earlier trials have been conducted to investigate biochemical markers of periodontal disease using combined saliva biomarkers and periodontal biofilm pathogens for the prediction and diagnosis of periodontal diseases (Ramseier et al., 2009, Kinney et al., 2011). More recently, it has been reported that saliva biomarkers are best for disease sensitivity, whereas the greatest disease specificity is found with GCF biomarkers.

In contrast, serum biomarkers do not show a significant role (Kinney et al., 2014). These findings were confirmed by Becerik et al. (2012) who reported that plasma cytokines unlike GCF cytokines are not related to inflammatory levels of periodontal disease.

It has been suggested that biomarker analysis throughout OTM and its association with obesity may need to be investigated in serum and GCF. The rationale behind the former is that some hormones, such as adiponectin and leptin are mainly produced by adipocytes and secreted in blood as the first transporter and investigated in serum (Saito et al., 2008, Furugen et al., 2008, Yamaguchi et al., 2010). Whilst the justification for GCF is that during OTM the supra-crestal gingival fibres are not totally destroyed, they are compressed or retracted to maintain PDL health during OTM (Redlich et al., 1996) and this will restrict the immediate movement of mediators through the gingival margin into the oral cavity (Junior et al., 2011). However, the components of GCF consequently pass to saliva, which is easier, less invasive and less expensive to collect and analyse. From the above information, in this study, the three different samples namely UWMS, GCF and serum, will be used to assess the levels of biomarkers.

### 3.1.1 Aims of the study

The specific aims of this preliminary study were:

1- Optimising the collection and processing of UWMS, GCF and serum; and
2- Detection of some biomarkers in the collected sample.

# 3.2 Optimisation of biomarker investigating techniques

## 3.2.1 Sample preparation

Sample collection including UWMS, GCF and serum was performed as described in Section 2.4. Total protein concentration was measured in UWMS, GCF and serum using NanoDrop (software V3.5.2) as described in Section 2.7. Sample preparation, electrophoresis and immuno-detection of proteins were performed as described in Sections 2.8 and 2.9.

## 3.2.2 Standard curve calibration

The reliability of the Periotron 8000 was tested using a known volume of nuclease free water applied to Periopapers and Sialostrips used for GCF and blood collection, respectively. This calibration was repeated twice and the Periotron readings were calculated and used to generate a calibration curves using polynomial regression formula $Y= 0.0001X^2 – 0.0053X$ for Periopapers (Figure 3.1) and $Y= 0.0001X^2 – 0.0006X$ for Sialostrips, where X is the Periotron reading and Y is the volume in microliters (µl) (Figure 3.2).

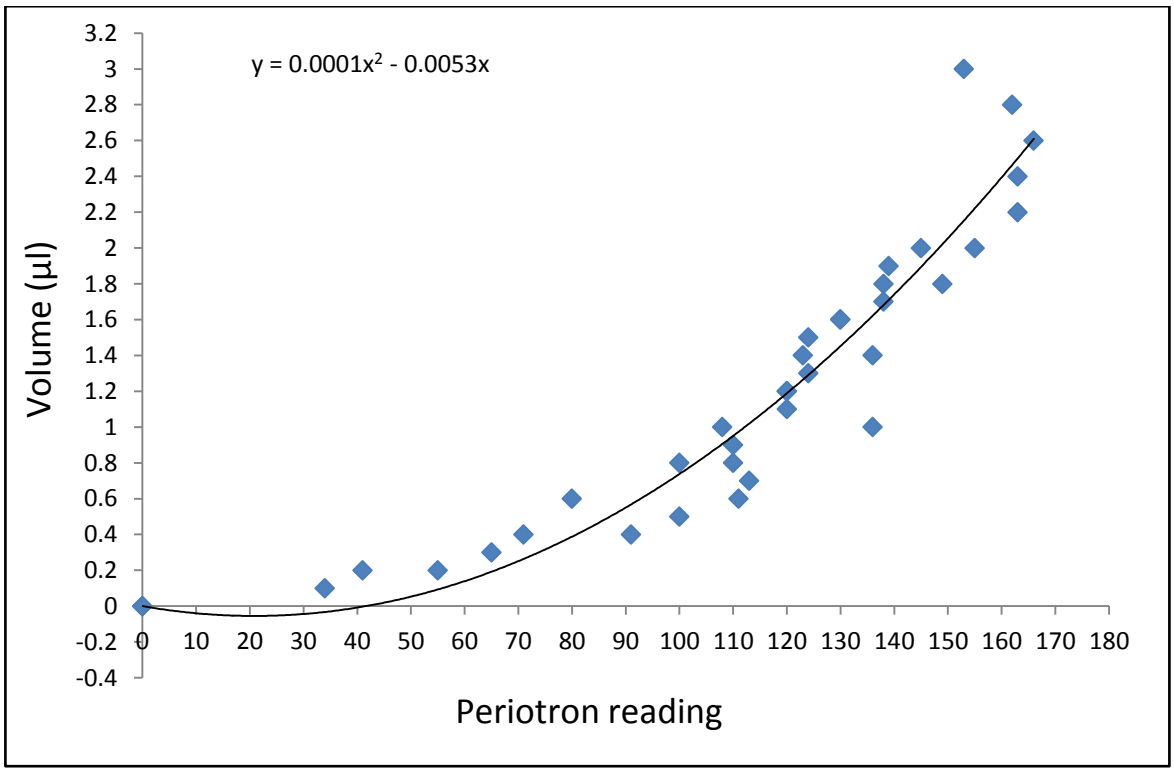



**Figure 3.1** The standard curve for Periotron 8000 using Periopaper.

X = Periotron readings; Y = Volume in microliters (µl)

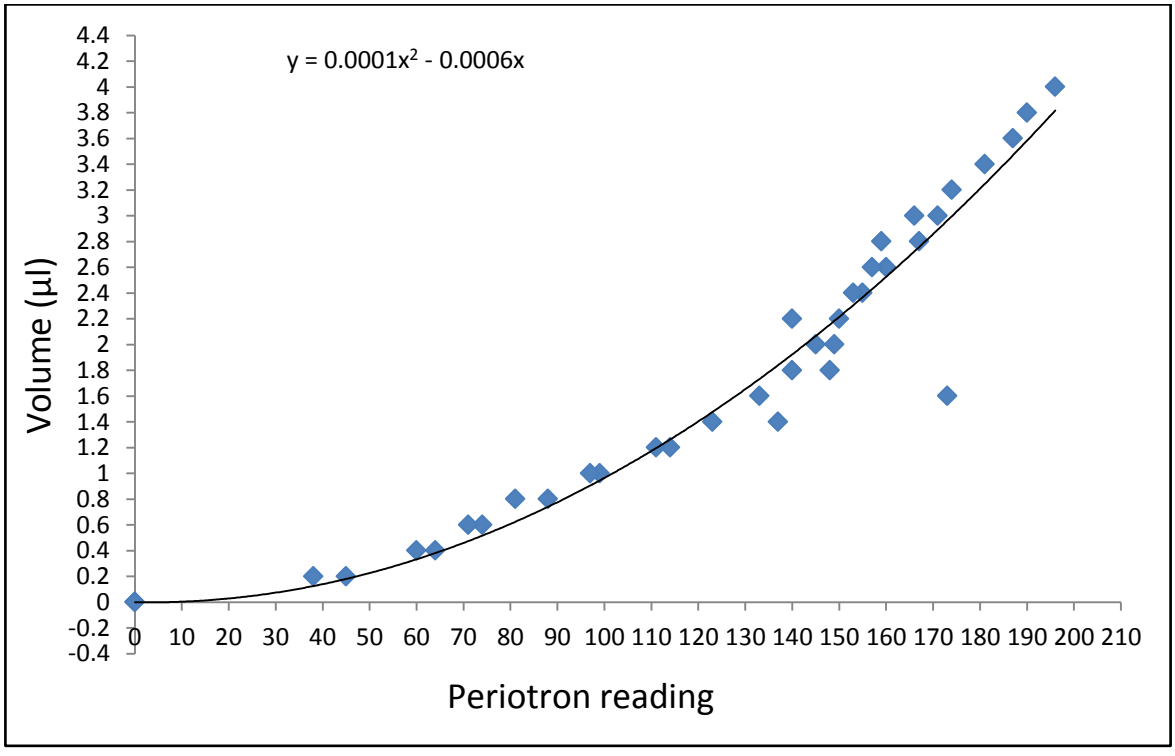



**Figure 3.2** The standard curve for Periotron 8000 using Sialostrip.

X = Periotron readings; Y = Volume in microliters (µl)

### 3.2.3 Centrifuging speed and time

The Periopapers and Sialostrips used to collect GCF and blood respectively were subjected to breakdown with centrifuging. Different centrifuge speeds and times were performed to optimise sample recovery.

GCF was eluted from Periopaper with 20 µl PBS, 1 µl was added to 60 Periopapers placed individually in the tubes, and divided into 4 groups of 15 according to the speed used (16300, 9200, 5900, 2300) g. Each group was subdivided into 3 subgroups according to the time used (5, 10, 15) minutes, as shown in Table 3.1. Some of the Periopapers were destroyed at 16300g at 3 tested times (5, 10 and 15) minutes. However, papers remained intact and were dry, when centrifuging at 9200 g for 5 and 10 minutes. Some papers broke down at 15 minutes. Moreover, intact Periopapers were obtained at 5 and 10 minutes centrifuging with 5900 g and at all times with 2300 g centrifuging, but these strips remained wet suggesting the sample may not have been fully eluted. Therefore, the optimal elution condition for Periopapers was centrifuging at 9200 g for 5 minutes.

The Sialostrips used to collect blood were also tested under the same conditions. Blood collected on the strip was eluted with 100µl PBS. The results in Table 3.2 show strips remained intact and almost dry when centrifuging for 5 minutes at 5900g. Higher speeds resulted in breaking down at all-time points. The strips remained wet at lower speed. Therefore, the optimal elution condition for Sialostrips was centrifugation at 5900g for 5 minutes.

**Table 3.1** Optimising centrifuging time and speed for the collected GCF using Periopaper. Each strip was eluted with 20µl PBS.

| No | Centrifuging Speed (g) | Centrifuging time (minutes) | Number of broken papers (out of 5 papers used) |
|---|---|---|---|
| 1 | 16300 | 5 | 1 |
| 2 | 16300 | 10 | 1 |
| 3 | 16300 | 15 | 2 |
| 4 | 9200 | 5 | 0 |
| 5 | 9200 | 10 | 0 |
| 6 | 9200 | 15 | 2 |
| 7 | 5900 | 5 | 0 * |
| 8 | 5900 | 10 | 0 * |
| 9 | 5900 | 15 | 1 |
| 10 | 2300 | 5 | 0 * |
| 11 | 2300 | 10 | 0 * |
| 12 | 2300 | 15 | 0 * |

*Wet strip

**Table 3.2** Optimising centrifuging time and speed for blood collection using Sialostrip. Each strip was eluted with 100µl PBS.

| No | Centrifuging Speed (g) | Centrifuging time (minutes) | Number of broken strips (out of 5 strips used) |
|---|---|---|---|
| 1 | 16300 | 5 | 1 |
| 2 | 16300 | 10 | 2 |
| 3 | 16300 | 15 | 3 |
| 4 | 9200 | 5 | 1 |
| 5 | 9200 | 10 | 1 |
| 6 | 9200 | 15 | 2 |
| 7 | 5900 | 5 | 0 |
| 8 | 5900 | 10 | 1 |
| 9 | 5900 | 15 | 1 |
| 10 | 2300 | 5 | 0 * |
| 11 | 2300 | 5 | 0 * |
| 12 | 2300 | 10 | 0 * |

*Wet strip

### 3.2.4 Identification of proteins

After loading 10 µl of UWMS, eluted GCF and eluted serum with equal amounts of total protein (3.9 µg) to SDS-PAGE gel electrophoresis, different stains such as Silver Stain, Coomassie Blue Stain and Periodate Schiff Stain (PAS) were used to identify different proteins in the samples. Some proteins are specifically noticed in one fluid, others are clearly diffused from serum to saliva (Figure 3.3).

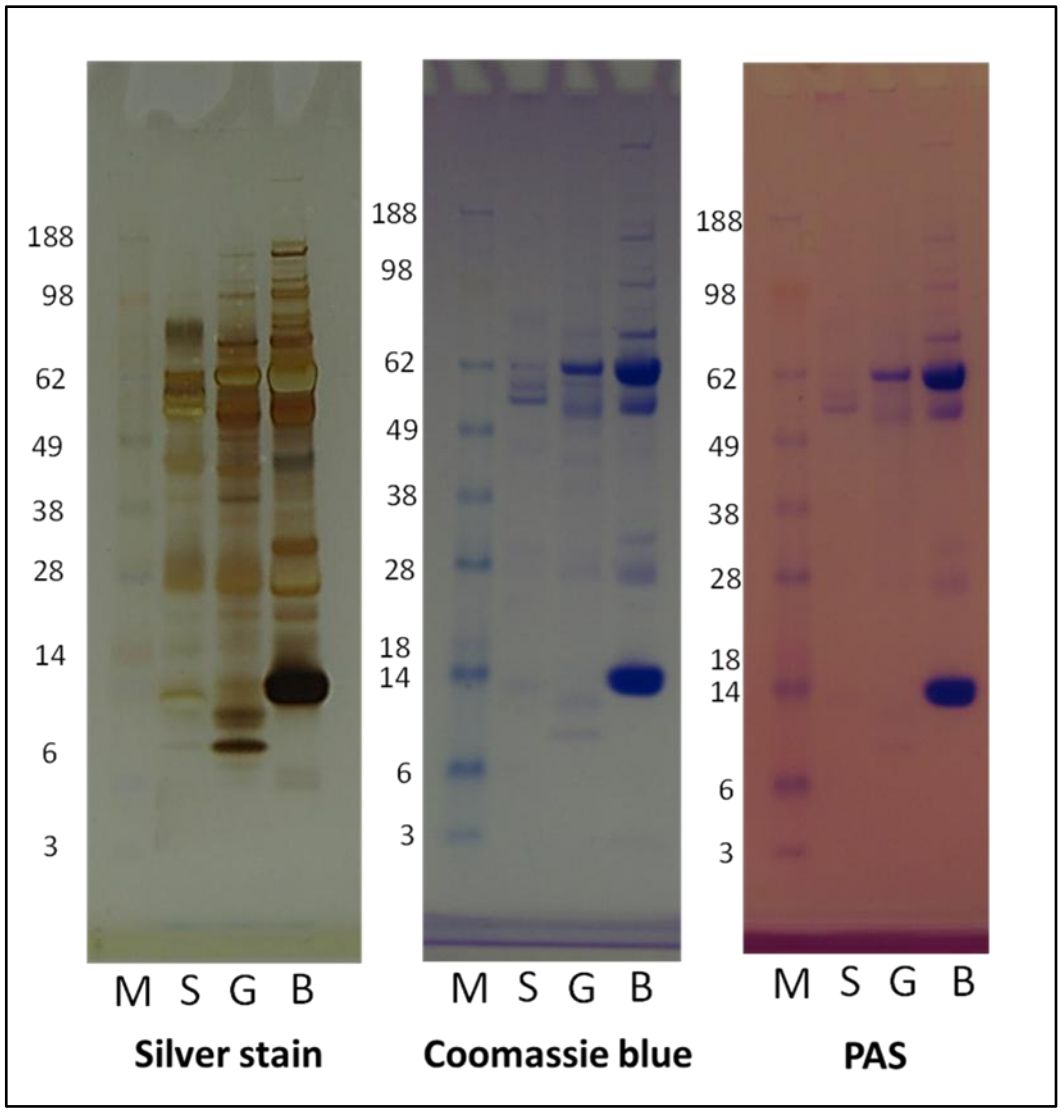



**Figure 3.3** SDS-PAGE gels represent the total protein in different bio-fluids. 10 µl of Unstimulated whole mouth saliva (S), gingival crevicular fluid (G) and serum (B) with different stains; PAS, Periodate Schiff stain M, molecular weight marker.

## 3.2.2 Detection of biomarkers

Certain biomarkers have been selected to optimise the proper collection of GCF, such as amylase; the diffusion of protein from serum to saliva via GCF, such as albumin; and the origin of others such as MMP9 and adiponectin.

### 3.2.2.1 Amylase enzyme

Saliva alpha amylase enzyme is entirely of salivary gland origin; around 80% of it is secreted by the parotid glands and the remainder by submandibular glands (Helmerhorst, 2012). Therefore, in the current study, it was used as a control to check the validity of GCF sample collection without saliva contamination. A total of 10 µl of UWMS and eluted GCF, with an equal amount of total protein (2.25 µg) was applied to SDS–PAGE gel. Proteins were transferred onto a nitrocellulose membrane by Western blot and immunoblotting. A sheep polyclonal amylase antibody (Abcam, UK) was used at a dilution of 1:1000 and 1:2000. Amylase was present in UWMS at both antibody dilutions and absent in GCF (Figure 3.4), which confirms the collected GCF was free from saliva contamination.

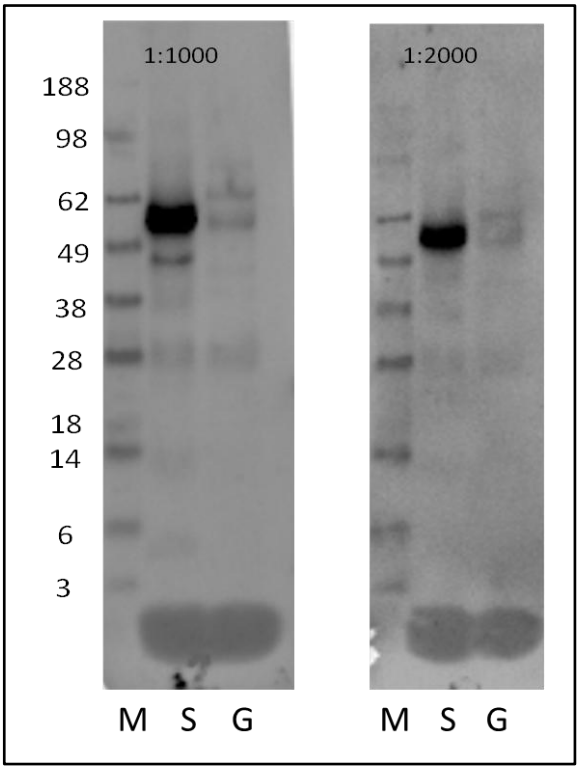



**Figure 3.4** Immunodetection of amylase different bio-fluid. Unstimulated whole mouth saliva (S) and gingival crevicular fluid (G); 1:1000, 1:2000: The dilutions of primary antibody; M, molecular weight marker.

### 3.2.2.2 Albumin

Albumin is abundant in serum; its level increases in GCF during inflammation as a result of increased vascular permeability (Giannopoulou et al., 1990) and to lesser extent due to an increased number of macrophages (White et al., 1980) and lymphocytes (Tunstall and James, 1974) during inflammation. 10 µl of UWMS, eluted GCF and eluted serum with an equal amount of total protein (4.8 µg) were loaded to SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane by Western blot and immunoblotting. Primary monoclonal anti-albumin antibody produced in mouse, 1:1000 dilution, was used and showed an intensive binding. More dilution of primary antibody to (1:10000) showed less bands in comparison to (1:1000) but multiple bands have also been seen. Further dilution of samples by loading 3 µl of UWMS (0.007 µg) to 7 µl distilled water, 2 µl of eluted GCF (0.003 µg) to 8 µl distilled water and 1 µl eluted serum (0.0014 µg) to 9 µl distilled water. Half amount (5 µl) of each sample was loaded in the SDS-PAGE gel and showed single albumin band at the same molecular weight indicated that blood is the origin of salivary albumin through GCF (Figure 3.5).

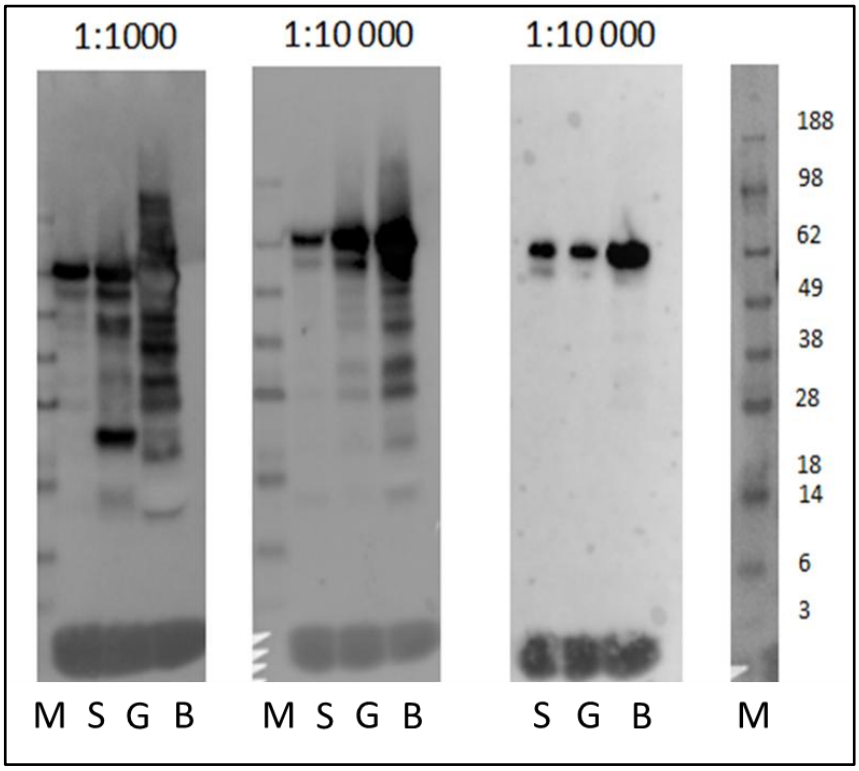



**Figure 3.5** Immunodetection of albumin in different bio-fluids. Unstimulated whole mouth saliva (S), gingival crevicular fluid (G) and serum (B); 1:1000, 1:10000: The dilutions of primary antibody; M, molecular weight marker.

### 3.2.2.3 Marix Metalloproteinase 9 (MMP9)

MMPs are a family of enzymes which play a vital role in PDL remodelling (Stamenkovic, 2003). A number of studies have reported an alteration of MMP quantity in human GCF as a consequence of applied orthodontic forces (Apajalahti et al., 2003, Cantarella et al., 2006). Others stated that the alteration in their GCF levels resulted either from biological changes within the gingival tissue (Junior et al., 2011) or in deeper periodontal tissues (Mogi et al., 2004). MMP9 is a member of this family, 10 µl of UWMS, eluted GCF and eluted serum samples at 3.0 µg total protein, were loaded in SDS-PAGE gel. Antihuman MMP9 primary antibody from goat (R&D Systems, Bio-Techne, UK) was used at 1:1000 dilutions. MMP9 was seen in GCF sample only. This has been confirmed by using (0.005, 0.01, 0.02 µg) standard Western blot. Control experiment also has been performed using only anti-goat secondary antibody (AbD Serotec), 1:2000 dilution, showed no MMP9 in all samples. In the conducted experiment, the immunoblotting of MMP9 showed a single band in GCF, but not in saliva or serum samples which recommend the local origin of MMP9 in the gingival sulcus (Figure 3.6).

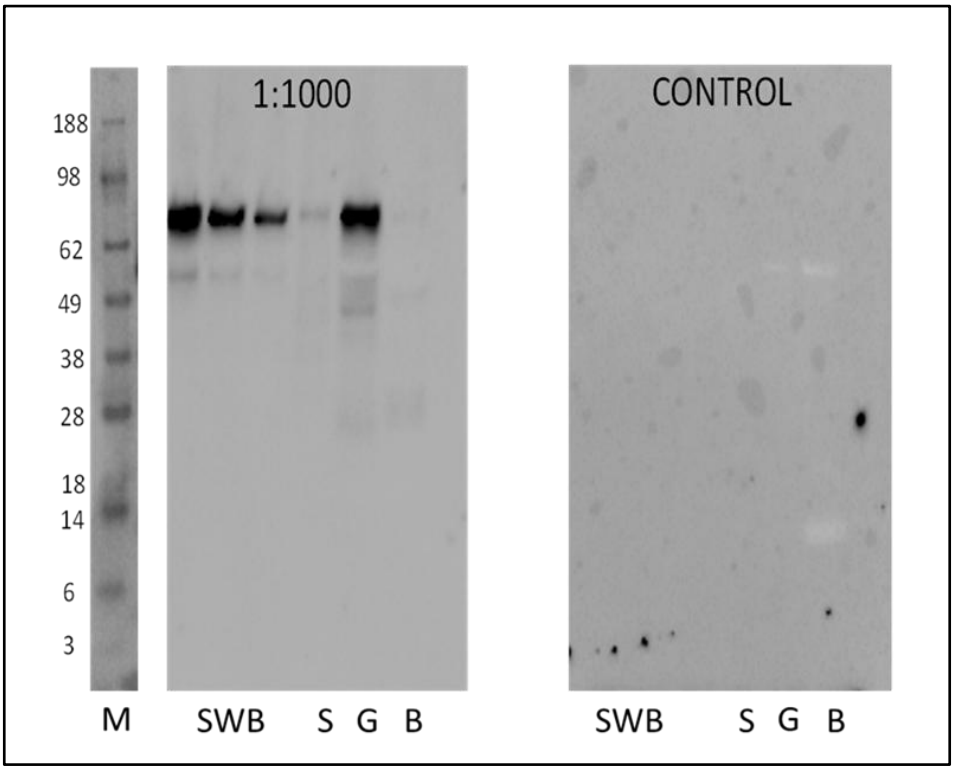



**Figure 3.6** Immunodetecion of MMP9 in different bio-fluids. Unstimulated whole mouth saliva (S), gingival crevicular fluid (G) and serum (B); 1:1000: Dilution of primary antibody; M, molecular weight marker; SWB: standard western blot purified protein (0.02 µg, 0.01 µg, 0.005 µg). Control means only secondary antibody was used without primary antibody.

### 3.2.2.4 Adiponectin

Adiponectin is an abundant serum protein, produced by adipocytes and demonstrates multiple functions by targeting different types of cells. It may exert many effects on obesity and related diseases (Turer and Scherer, 2012), therefore it is speculated that it can be affected by orthodontic treatment of obese patients.10 µl of UWMS, eluted GCF and eluted serum with an equal amount of total protein (4.0 µg) were applied to a SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane by Western blot and immunoblotting. Anti-adiponectin primary antibody produced in rabbit (sigma, UK) at dilution 1:2000 was applied. Adiponectin present in serum sample around 30 kDa, smaller adiponectin protein band was also found at higher molecular weight. In UWMS band was seen at molecular weight 49-62 kDa. However, it was absent in GCF. Moreover, no adiponectin protein was found in control blots using only anti rabbit secondary antibody at dilution 1:2000 (Dako, UK) (Figure 3.7).

A single band in UWMS was seen at a different molecular weight than those in serum. Thus further investigation is required to explore this protein and its activity.

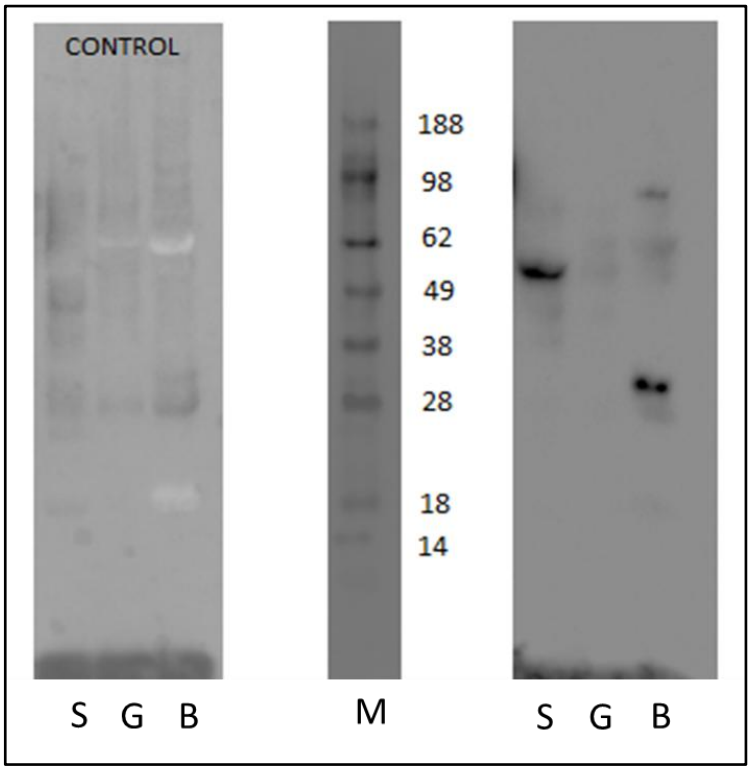



**Figure 3.7** Immunodetection of adiponectin in different bio-fluids. Unstimulated whole mouth saliva (S), gingival crevicular fluid (G) and serum (B). Control means only secondary antibody was used without primary antibody; M, molecular weight marker.

# 3.3 The origin of salivary adiponectin

## 3.3.1 Background

Adiponectin is a protein produced and secreted mainly by adipocytes; it exists as a full-length protein and/or globular adiponectin. This cleavage is mediated by a leukocyte elastase, secreted by activated monocytes and/or neutrophils (Tilg and Moschen, 2006). It has multiple biological activities that are mediated by its three receptors, AdipoR1, AdipoR2 and T-cadherin.

Currently, adiponectin is predominantly detected in blood samples. A few studies have investigated adiponectin levels in saliva as an easier and non-invasive sampling route that would be useful for assessing adiponectin in many clinical studies; however the results of these studies are confusing. One study found increases in saliva levels of adiponectin when samples were diluted. The authors explained these findings by suggesting that there are potentially assay inhibitory factors in saliva that may be prone to greater dilution effects than the adiponectin protein. Thus, dilution of saliva results in an apparent increase in adiponectin as the inhibitory factors cease to be effective at a greater rate (Akuailou et al., 2013).

Another study observed the band pattern of adiponectin in human serum and saliva by Western immunoblotting under non-reducing conditions. They found two bands in serum (medium molecular band at 150 kDa and higher molecular weight band at 250 kDa), whereas, only one band in saliva which was detected - above the higher molecular band that seen in serum - called super higher molecular band (Lin et al., 2014). Furthermore, most of the conducted studies mentioned a very low level of adiponectin in human saliva relative to plasma (Toda et al., 2007, Toda and Morimoto, 2008, Akuailou et al., 2013); however, the nature and precise forms of adiponectin in human saliva are not well understood.

### 3.3.1.1 Aims and objectives

The main aim of the present study was to verify the origin of adiponectin in saliva.

In order to address this aim the following steps were undertaken:

1- Detection of adiponectin using different assays such as Western blot and immunoblotting, ELISA and magnetic Luminex multiplex array assay; and
2- Mass spectrometry (proteomics) to identify the proteins.

This study hypothesized that adiponectin can be detected in saliva and this is derived from the circulation.

## 3.3.2 Methods

The study was approved by the Human Research Ethics Committee of King's College London (BDM/14/15-8, November 2014). Informed written consent was obtained from all participants.

### 3.3.2.1 Participants and sampling

The participants in this study were recruited as mentioned in Section 2.4 number 1, including 20 normal body weight (BMI = 18.5-25) healthy adult volunteers (10 males and 10 females) with a mean age of 29 years (SD=7.1) ranged between 19-44 years.

From each participant UWMS, GCF and serum were collected as described in Section 2.4.

### 3.3.2.2 Samples analyses

Detection and quantification of adiponectin were undertaken by Western blot and immunoblotting as described in Section 2.9. Samples were loaded with reducing and heating conditions using 5% NuPage lithium dodecyl sulphate sample buffer (LDS, Invitrogen), 10% 0.5 M dithiothreitol (DTT) and heated for 3 minutes at 100 °C. In addition, samples were loaded under non-reducing and non-heating conditions in which additional 2 µl distilled water was added instead of DTT without heating the samples.

In order to confirm the effect of heat denaturation and reducing conditions on the detected protein in saliva, UWMS samples from randomly selected 5 participants were similarly loaded in another gel for electrophoresis with and without heating and reducing conditions. The protein was transferred to nitrocellulose membrane by Western blot and immunoblotting for adiponectin detection, as described in Section 2.9.

Then purified adiponectin and UWMS from the 20 participants were loaded under reducing and heating conditions in other gels for Western immunoblotting to detect the protein in UWMS of all subjects, as described in Section 2.9.

Additionally, ELISA as described in Section 2.12, and magnetic Luminex screening assay as described in Section 2.13 were undertaken to quantify adiponectin in UWMS,

GCF and serum of 20 healthy adults by the first and 19 healthy adults by the second assay because of limited room in Luminex plate.

Under heating and reducing conditions, purified adiponectin standard (Recombinant human adiponectin (NSO-derived)/Acrop30, CF, R&D Systems, Bio-Techne, UK), UWMS, eluted GCF and eluted serum were loaded in the same gel twice, as described in Section 2.8. After electrophoresis, proteins in one half of gel were transferred to nitrocellulose membrane by Western blot for detection of adiponectin by immunoblotting, as described in Section 2.9. Proteins of interest were identified by immunoblotting then cut from the second half and sent for proteomic analysis to detect the proteins, as described in Section 2.11.

### 3.3.2.3 Tissue culture

Tissue culture was performed to assess the biological activity of the existing protein (pseudo-adiponectin) in parotid saliva.

The human gingival fibroblast cell lines (HGF-1 ATCC CRL-2014$^{TM}$ Homo sapiens, human gingival biopsy, normal, UK) expressing adiponectin receptors were grown in Dulbecco's modified eagles medium (DMEM) (Sigma, UK) containing 4500 mg/l glucose with 10% fetal bovine serum (FBS), 1% L-glutamin and 1% penicillin-streptomycin (P/S) (Sigma-Aldrich, Dorset, UK). Cells were seeded into 12-well plates at a density of 1.8 x $10^5$ cells in each well from a 75 cm four flasks which was split when approximately 85-90% confluent.

Following 18 hr of pre-incubation without or with either 1 ml adiponectin (10 µg/ml) or 1 ml of parotid saliva, cells were treated with either 1 ml of IL-1β (0.5 ng/ml) or of 1 ml of suspended salivary bacteria for 12 hr. At the end of the incubation periods, the supernatants were collected and the concentrations of IL-6 and IL-8 cytokines were measured in triplicate using magnetic Luminex performance assay kits for cytokines (R&D Systems, Bio-Techne; Abingdon, UK) according to the manufacturer's instructions.

Parotid saliva for tissue culture assay was prepared as mentioned in Section 2.5; 250 µl of parotid saliva added to 750 µl of DMEM. Salivary bacteria suspension was prepared by collecting UWMS for 5 minutes, centrifuged at 500 rpm for 10 minutes. The supernatant was aliquoted as 1 ml in each 1.5 ml Eppendorf (Alpha Laboratories, UK) and centrifuged at 9200 g for 10 minutes. The supernatants were discarded leaving salivary bacteria pellets

which were suspended in 1 ml of DMEM, then 500 µl of the suspended salivary bacteria added to 500 µl of DMEM to treat the cells.

### 3.3.2.4 Study size

A previous study investigated the levels of the GCF biomarkers MMP8, MMP9, IL-1β, OPG and CRP as a predictor of periodontal disease progression (Kinney et al., 2014). They found differences in the levels of GCF biomarkers between participants with stable periodontitis and those with progressing periodontitis and the mean effect size was 2.77. Using this effect size (assuming a significance level of 0.05 and power of 0.80) it was calculated (Gpower 3.1.2) that a sample size of 8 would be sufficient to detect a significant difference in the biomarkers between the obese and the normal weight groups. However, to allow for dropouts and a weaker effect size for biomarkers in saliva, a total sample size of 40 (20 in each group) was recruited.

In this study one group of 20 normal weight participants was included to identify the origin of adiponectin in saliva, whereas 20 normal weight and 20 obese participants were included in Chapter 4 to compare the levels of the selected biomarkers between normal weight and obese subjects.

### 3.3.2.5 Statistical analysis

Descriptive statistics were used to summarise the outcome variables. Parametric and non-parametric analyses were carried out after checking for the normality assumptions using the Shapiro-Wilk test of normality.

The non-normally distributed data of adiponectin concentrations in UWMS, GCF and serum groups were analysed using Kruskal –Wallis test. One-way analysis of variance (ANOVA) was used to analyse the normally distributed data of the IL-6 and IL-8 concentrations in the supernatants of human gingival fibroblast. If the overall significance was achieved, Post Hoc analysis was carried out with Bonferroni step-down correction. A p-value of $<0.05$ was considered statistically significant. All statistical analyses were done using SPSS (BM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 23; Armonk; New York; IBM;Corp).

## 3.3.3 Results

The clinical parameters of the subjects enrolled in the present study are shown in Table 3.3.

**Table 3.3** Demographics and clinical characteristics of the volunteers participated in adiponectin study.

| Parameters | Subjects |
|---|---|
| Number | 20 |
| Age (years): Mean (SD) | 29 (7.1) |
| Gender: male / female | 10 / 10 |
| UWMS flow rate (ml/min): Mean (SD) | 0.56 (0.21) |
| GCF flow rate (µl/min): Mean (SD) | 0.6 (0.1) |

UWMS, unstimulated whole mouth saliva; GCF, gingival crevicular fluid.

Under non-reducing and non-heat denaturing conditions, purified adiponectin resolved to around 55 kDa, whereas several different molecular weights variants of adiponectin were seen in serum as 67 kDa, 56 kDa and 28 kDa. In GCF no adiponectin band was detected. However, in UWMS an immuno-reactive band was found at molecular weight, around 50 kDa. Under reduced conditions, purified adiponectin reduced to around 30 kDa, whereas the single band seen in UWMS remained at 50 kDa. The 2 distinct bands in serum possibly corresponding to the trimer (67 kDa) and the monomer band resolved to a single band (30 kDa) (Figure 3.8).

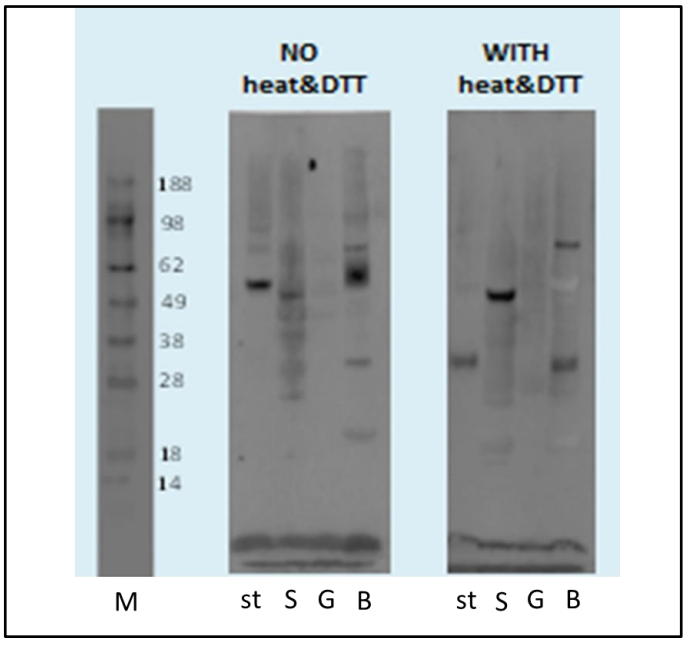



**Figure 3.8** Immunodetection of adiponectin in different bio-fluids. Standard purified adiponectin (st), unstimulated whole mouth saliva (S), gingival crevicular fluid (G), serum (B); M, molecular weight marker; DTT: dithiothreitol.

UWMS of 5 subjects were investigated with and without heating and reducing conditions showed positive bands at around 50 kDa, which are not related to any of multimer adiponectin in serum. Generally these bands showed no change in their molecular weight after reducing and heating conditions but they appeared stronger (Figure 3.9). The same bands have been seen (50 kDa) in UWMS of 20 participants, under reducing and heat denaturation conditions, which are away from purified adiponectin bands (30 kDa) with 0.47 µg/ml band intensity (Figure 3.10).

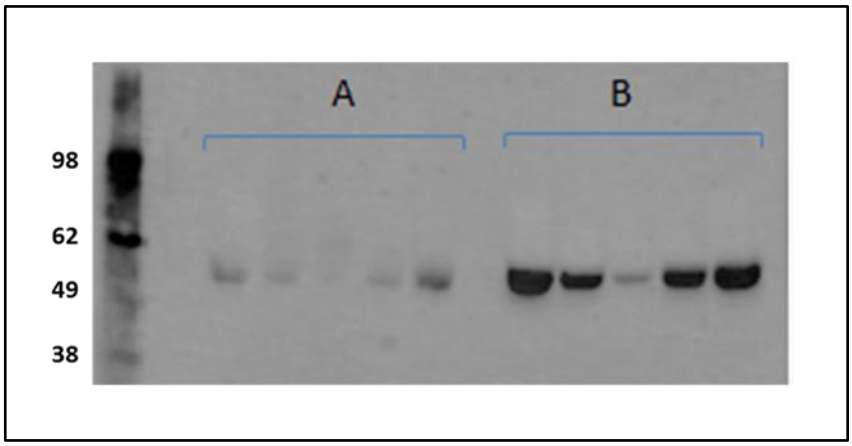



**Figure 3.9** Immunodetection of adiponectin in unstimulated whole mouth saliva of 5 subjects. (A) Without heating and reducing conditions. (B) With heating and reducing conditions.

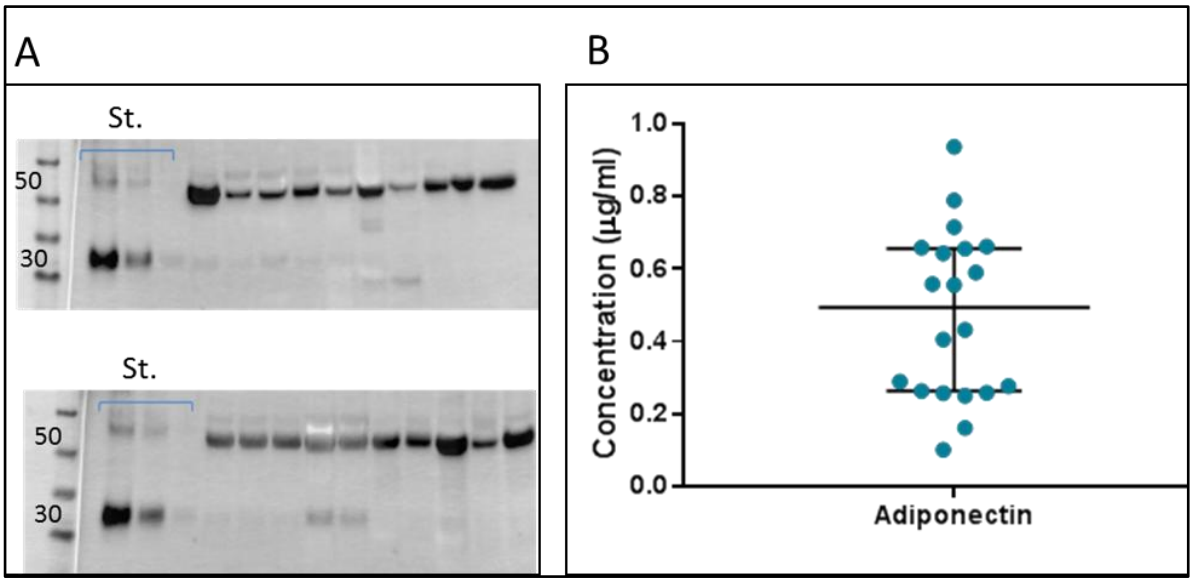



**Figure 3.10** Immunodetection of adiponectin in unstimulated whole mouth saliva of 20 subjects with heating and reducing conditions. (A) Immunoblotting (St, standard purified adiponectin). (B), Scatterplot showing band intensity relative to purified adiponectin (median with 95%CI).

ELISA was used to assess the levels of adiponectin in UWMS, GCF and serum of 20 healthy subjects. As shown in Figure 3.11, the highest level of adiponectin was found in serum with a mean of 475 µg/ml (SD, 181), less in GCF with a mean of 59 µg/ml (SD, 71) and the least in UWMS with a mean of 0.3 µg/ml (SD, 0.4) with high significant difference ($p<0.001$).

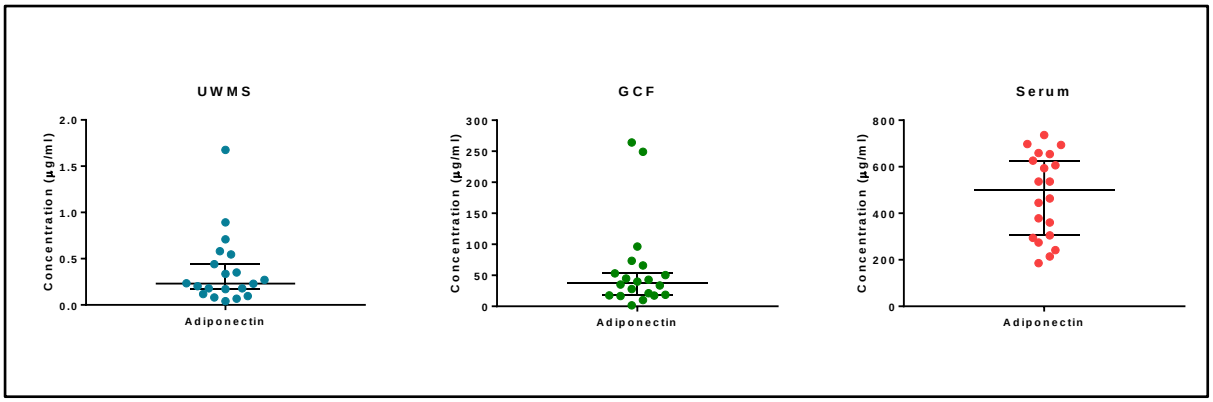



**Figure 3.11** Scatterplots showing the levels of adiponectin (median with 95%CI) in different bio-fluids by ELISA. Unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 20 healthy adults; ***, significant difference ($p<0.001$).

Magnetic Luminex screening assay has been carried out to measure the level of adiponectin in UWMS, GCF and serum of 19 normal healthy subjects. The highest level of adiponectin found in serum with a mean of 66.3 µg/ml (SD, 49.4), less in GCF with a mean of 3.8 µg/ml (SD, 2.3) and the least in UWMS with a mean of 0.05 µg/ml (SD, 0.02) with high significant difference ($p<0.001$) (Figure 3.12).

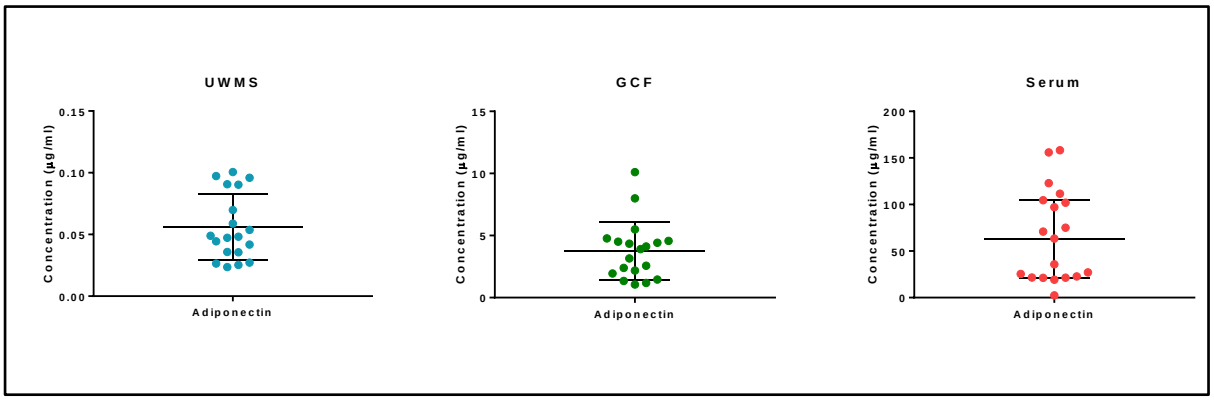



**Figure 3.12** Scatterplots showing the levels of adiponectin (median with 95%CI) in different bio-fluids by Luminex. Unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 healthy adults; ***, significant difference p<0.000.

Proteomic analysis for the positive bands seen in purified adiponectin, UWMS and serum showed that adiponectin was detected in the purified adiponectin at very low numbers of detected peptides, and with a low probability in serum and no adiponectin in UWMS (Appendix 3 and 4).

To test the biological activities of the salivary pseudo adiponectin protein in comparison to purified adiponectin, we measured the effect of adiponectin and parotid saliva on the levels of IL-6 and IL-8 expressed by a human gingival fibroblast cell line (HGF-1) stimulated by salivary bacteria and pro-inflammatory cytokine IL-1β. Adiponectin and parotid saliva significantly reduced the levels of IL-6 and IL-8 induced by IL-1β and salivary bacteria. Furthermore, adiponectin and parotid saliva showed comparable reduction in the levels of IL-6 and IL-8 induced by salivary bacteria; however, the anti-inflammatory effect of parotid saliva was significantly greater than adiponectin on IL-6 and IL-8 induced by IL-1β (Figures 3.13 and 3.14).

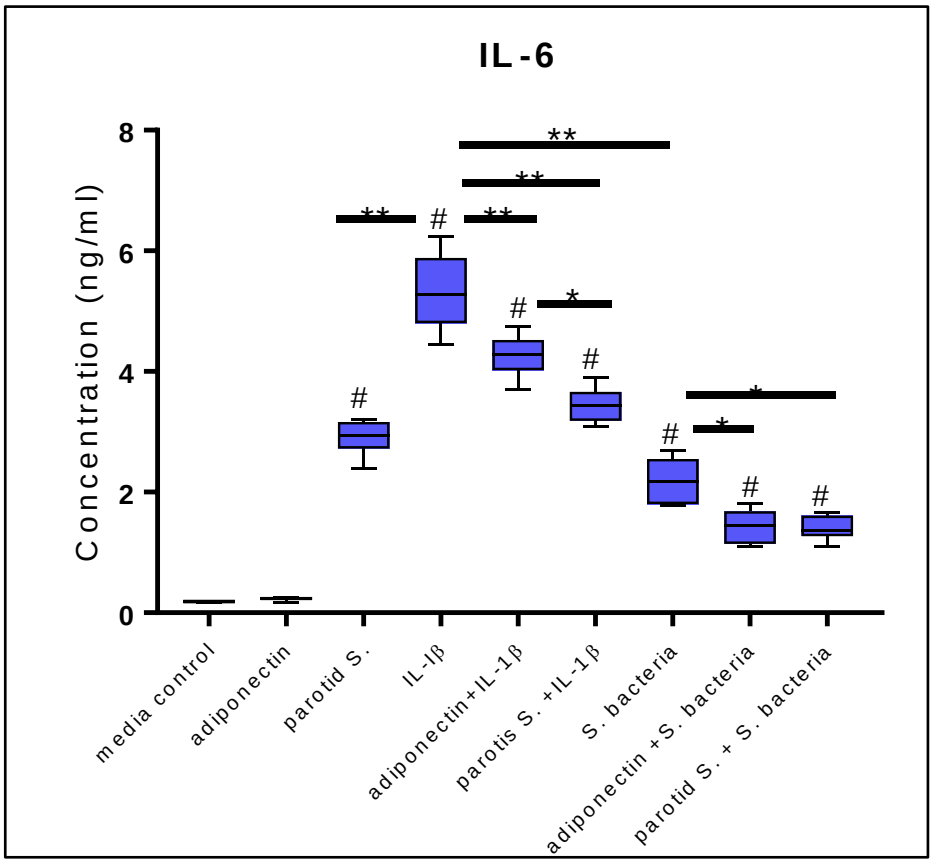



**Figure 3.13** Box plots showing the effect of adiponectin and parotid saliva on the levels of IL-6 expressed by human gingival fibroblast cell line (HGF-1) stimulated by salivary bacteria and IL-1β; *, significant differences $p<0.01$; **, significant differences $p<0.001$; #, significant differences comparing to none and adiponectin $p<0.001$, vertical whiskers indicate minimum to maximum values.

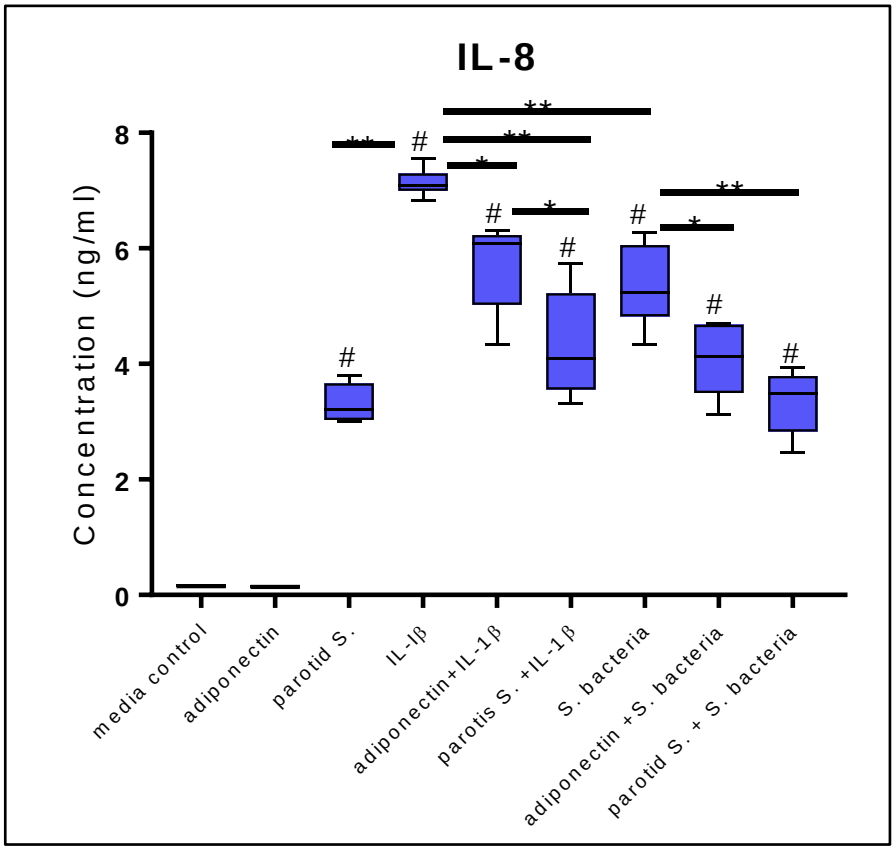



**Figure 3.14** Box plots showing the effect of adiponectin and parotid saliva on the levels of IL-8 expressed by human gingival fibroblast cell line (HGF-1) stimulated by salivary bacteria and IL-1β; *, significant differences p<0.01; **, significant differences p<0.001; #, significant differences comparing to none and adiponectin p<0.001, vertical whiskers indicate minimum to maximum values.

## 3.3.4 Discussion

The main aim of this study was to verify the source of salivary adiponectin. Adiponectin, is mainly produced by adipocytes and secreted into blood, to date this has mostly been investigated in blood (Furugen et al., 2008, Saito et al., 2008, Yamaguchi et al., 2010). GCF composition often reflects the concentration of metabolites in blood and its collection is less invasive than blood. However, the components of GCF consequently pass to saliva, which is easier, less invasive and less expensive to collect and analyse. One study found that adiponectin could be produced by salivary gland epithelial cells (Katsiougiannis et al., 2006) and might play a protective role during inflammation of the salivary gland (Katsiougiannis et al., 2010). From the above information, in this study the three different samples namely UWMS, GCF and serum, were used to assess the sources of adiponectin.

The results of the immunoblotting confirmed by previous studies showed that SDS-PAGE under non-reducing and non-heat denaturing conditions shows several different molecular weights of human serum adiponectin, designed as low molecular weight including monomer, dimer and trimer (Waki et al., 2003). The same study showed that with a reducing agent adiponectin converted into a trimer of 67 kDa, whereas with 10 minutes heat at 95 °C it was converted into a dimer of 56 kDa and a monomer of 28 kDa, with both heat denaturing and reducing conditions all adiponectin forms were converted into monomer. In this study, not all adiponectin structures were broken down into monomer, which could be related to different type and concentration of reducing agent used. For instance, Waki et al. (2003) utilized both beta mercaptoethanol and DTT as a reducing agent. Moreover, adiponectin monomers and trimers can bind with albumin showing fractions at 90-100 kDa (Hada et al., 2007).

A single band that has been seen in UWMS at molecular weight of around 50 kDa and this is similar to the bands seen in immunoblotting assays of adiponectin in saliva (Akuailou et al., 2013). However, this band is not necessarily adiponectin, as in the present study, the band in UWMS occurred at different molecular weight than that of serum and purified adiponectin with and without heating and reducing conditions. Moreover, it did not show any change in molecular weight after heating and reducing conditions as purified adiponectin and adiponectin in serum did, that confirmed by immunoblotting of UWMS from other 5 subjects. Additionally, under heating and reducing conditions, same bands (50 kDa) were seen in UWMS of all participants, which are at different molecular weight of purified adiponectin and may indicate that this protein is unlikely to be adiponectin.

Although previous study showed adiponectin receptors R1 and R2 expressed in human PDL cells and gingival fibroblasts (Iwayama et al., 2012), under reducing and heat denaturation conditions, no clear band could be found by immunoblotting the GCF sample in this study.

Both ELISA and Luminex multiplex assays showed the highest concentration of adiponectin in serum and the lowest in UWMS, this is well matched with the outcome of other studies, which stated that human salivary adiponectin level is very low (0.37-6.42 ng/ml) in comparison to that of plasma (0.58-12.40 µg/ml) (Toda et al., 2007). In addition, Toda and Morimoto (2008) reported that adiponectin level in plasma (8.94-13.68 µg/ml) is higher than its level in saliva collected in test tube and in salivette (0.69-1.41 ng/ml, 0.71-0.86 ng/ml) respectively . In the present study, the value of the detected protein in UWMS

by ELISA is more or less approximate to values of pseudo adiponectin protein detected by immunoblotting, which is 7-8 folds the levels of adiponectin measured in UWMS by Luminex.

Furthermore, proteomic analysis showed adiponectin was detected in the purified adiponectin (standard) at very low numbers of detected peptides, and with a low probability in serum but not in UWMS. These findings indicate that the positive results of adiponectin in saliva might come from other proteins, which have the same anti-inflammatory effect as adiponectin, as confirmed by our tissue culture experiments when both purified adiponectin and parotid saliva reduced the levels of IL-6 and IL-8 expressed by human gingival fibroblast as a result of inflammation induced either by IL-1β or salivary bacteria. These findings agree with a previous study showing that adiponectin reduced the production of IL-6 and IL-8 expressed by human gingival fibroblasts (Iwayama et al., 2012). Further study is required to identify this specific protein.

### 3.3.5 Conclusion

Salivary adiponectin has a small contribution from serum via the GCF but there also appears to be a contaminating protein, which reacts with several antibodies in Western blots and ELISA, which is unlikely to be adiponectin.

# Chapter 4 Effects of obesity on biochemical changes in saliva, GCF and serum with and without orthodontic treatment: a cross sectional study

This chapter includes two main cohorts; the first cohort is the control group which has normal weight and obese adults without orthodontic treatment; and the second cohort has normal weight and obese adults underlying fixed-appliance orthodontic treatment with 0.019 x 0.025-inch stainless steel archwires in upper and lower arches.

## 4.1 Control cohort

### 4.1.1 Introduction

The accumulation of fat in obesity results in serious health complications, increasing the risk of significant disease and morbidity. Adipose tissue does not only represent the region of fat storage in the body but also acts as an endocrine organ that is able to secrete multiple immunomodulatory proteins known as adipokines (Exley et al., 2014). Adipokines such as adiponectin, leptin, resistin and omentin can be defined as molecules secreted by adipose tissues that participate in the regulation of appetite and glucose homeostasis as well as modulating the immune response (Tilg and Moschen, 2006). Physiologically, adipose tissue can secrete constitutive levels of adipokines, which have been shown to have both anti-inflammatory properties, such as adiponectin; and pro-inflammatory activities, such as leptin, resistin, as well as pro-inflammatory cytokines such as TNF-α and IL-6. In obese subjects, adipocytes and associated immune cells are known to increase the expression of pro-inflammatory molecules and reduce anti-inflammatory, resulting in a chronic low-grade inflammatory state (Nakamura et al., 2014, Fantuzzi, 2005). The increased circulatory level of inflammatory mediators, particularly IL-6 has been associated with hepatocyte stimulation to synthesize and produce a low-grade systemic inflammation marker CRP (Zhang et al., 2009), which has been classified as an essential cause of many illness

conditions including metabolic syndrome (Bassuk et al., 2004), atherosclerosis and coagulation (Adar et al., 2015), coronary heart disease (Danesh et al., 2008), cancers and metastases (Zhou et al., 2012), and other health conditions like depression (Musselman et al., 2001).

A number of biomarkers for obesity have been studied in the bloodstream. Amongst these, leptin exerts a role in both immunity and inflammation, stimulating the secretion of pro-inflammatory mediators (Procaccini et al., 2012) as well as controlling appetite. Resistin, which has pro-inflammatory effects, involved in several inflammatory diseases (Park and Ahima, 2013); whilst other cytokines, such as TNF-α and IL-6, are produced by monocytes and macrophages, but are up-regulated by adipose tissue (Nakamura et al., 2014). In contrast, adipokines with anti-inflammatory activity such as adiponectin can strongly induce the production of anti-inflammatory cytokines and down-regulate the activity of pro-inflammatory mediators (Nigro et al., 2014, Fantuzzi, 2005). In addition to the blood, these and other biomarkers have also been measured using alternative biological media. Saliva is a good diagnostic tool offering an excellent alternative to other body fluids for investigative purposes (Pfaffe et al., 2011). GCF is a transudate of the interstitial fluid, since serum is the main origin of the aqueous portion of GCF, its composition is highly modified by both local and systemic conditions (Kavadia-Tsatala et al., 2001). The composition of GCF can therefore reflect the systemic as well as local conditions in humans. Venous blood is generally considered to be the best body fluid for the evaluation of systemic processes. However, the collection of blood involves potential risks to subjects, such as bruising, transient discomfort and infection at the venepuncture site. Moreover blood collection needs a trained professional to collect it, as well as specific handling and collection vessels. It is less favourable in research involving children, as well as in other research subjects for whom venous access is difficult (elderly or critically ill) (Williamson et al., 2012). The substitution of other body fluids, such as saliva and GCF would greatly improve ease of sample collection. However, analysis of biomarkers in these fluids from humans has not been widely researched.

#### 4.1.1.1 Aims and objectives

The specific aims of this study were to investigate the effect of obesity on selected biomarkers and to measure these biomarker levels in UWMS, GCF and serum.

To address these aims the following steps were undertaken:

1. Comparing the levels of biomarkers in obese and normal weight subjects; and
2. Measuring the levels of biomarkers in UWMS, GCF and serum.

The hypothesis was that obesity does not change the levels of the selected biomarkers.

## 4.1.2 Methods

The study was approved by the Human Research Ethics Committee of King's College London (BDM/14/15-8, November 2014). Informed written consent was obtained from all participants.

### 4.1.2.1 Participants and sampling

The 48 healthy adult volunteers (20 males and 28 females) were recruited as described in Section 2.4, number 1. For all participants, plaque levels and gingival health were considered clinically acceptable. Height and weight were measured to calculate BMI as described in Section 2.2.1. According to BMI, 6 females were classified as overweight (BMI = 25 - 30) and 2 were underweight (BMI<18.5) and were excluded from the study. The sample consisted of 20 normal weight (BMI =18.5 - 25) (10 males and 10 females) with a mean age of 29 years (range 19-44 years) and 20 obese (BMI ≥30) (10 males and 10 females) with a mean age of 30.95 years (range 18-45 years) (Figure 4.1).

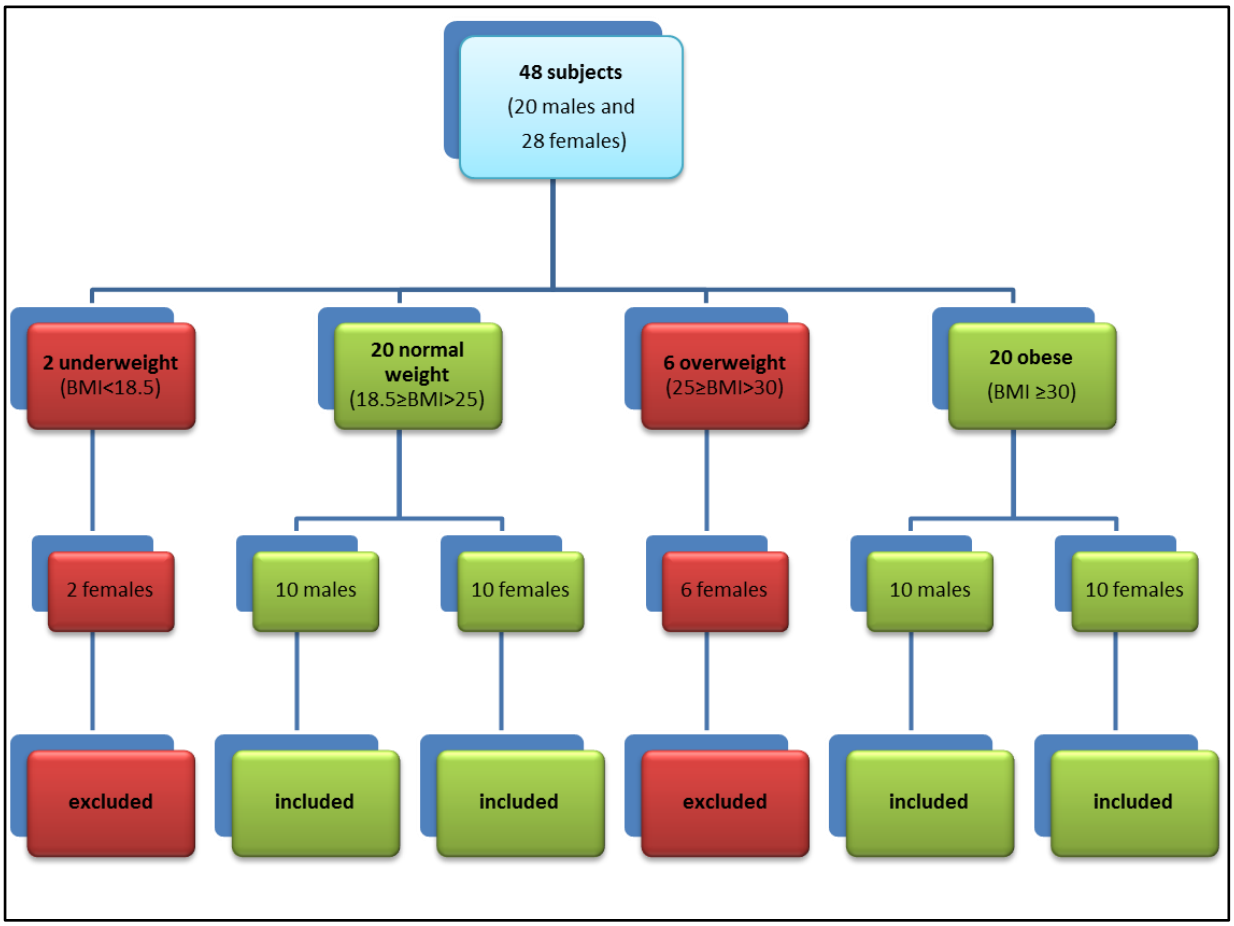



**Figure 4.1** Diagram for sample distribution of control cohort.

From each individual UWMS, GCF and serum were collected, as described in Section 2.4. UWMS and GCF flow rate were measured as mentioned in Section 2.6. UWMS were analysed for albumin and secretory immunoglobulin A (sIgA) contents using Western blot and immunoblotting as described in Section 2.9.

In addition, UWMS was analysed for gelatinase using 10% zymogram gelatin gels electrophoresis as described in Section 2.10. The levels of selected biomarkers were determined using a magnetic Luminex screening assay (R&D Systems, Bio-Techne; Abingdon, UK) for only 19 participants from each group because of the limited room in the Luminex plate, as described in Section 2.13.

### 4.1.2.2 Study size

The sample size calculation was based upon a previous study (Kinney et al., 2014) as described in Section 3.3.2.4.

### 4.1.2.3 Statistical analysis

Descriptive statistics were used to summarise outcome variables. Parametric and non-parametric analyses were carried out after checking for the normality distribution using Shapiro-Wilk test of normality. Kruskal-Wallis test was used to analyse the not normally distributed data of the three gelatinase bands measured. Mann Whitney U test was used to compare the not normally distributed data of biomarkers' concentrations and sIgA concentrations between the normal weight and obese groups. Independent t-test was used to compare the normally distributed data of albumin concentration, age, BMI and UWMS and GCF flow rates in both groups. All statistical analyses were done using SPSS (BM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 23; Armonk; New York; IBM;Corp).

## 4.1.3 Results

The clinical parameters of the subjects enrolled in the present study are shown in Table 4.1. Age did not significantly differ between obese and normal weight groups (p=0.380).

**Table 4.1** Clinical characteristics of participants in control cohort study.

| Parameters | Normal weight | Obese |
|---|---|---|
| Number | 20 | 20 |
| Age (years): Mean (SD) | 29 (7.1) | 30.9 (6.9) |
| Gender: male / female | 10 / 10 | 10 / 10 |
| BMI ($kg/m^2$): Mean (SD) | 21.5 (1.6) | 32.2 (2.5)* |

SD, standard deviation; BMI, body mass index; *, significantly higher (p<0.0001) using independent t-test.

### 4.1.3.1 UWMS and GCF flow rate

The flow rate of saliva is frequently altered in different disease states. Additionally, periodontal health status and mechanical stimuli can modify GCF flow rate. We therefore measured the flow rate of UWMS and GCF in our normal weight and obese groups.

The UWMS flow rate was comparable for both obese and normal weight groups (p=0.146), whereas the GCF flow rate was significantly higher in the obese group compared to normal weight (p=0.0001) (Table 4.2).

**Table 4.2** UWMS and GCF flow rate of 20 obese and 20 normal weight adults of control cohort study.

| | **UWMS flow rate** | | **GCF flow rate** | |
|---|---|---|---|---|
| | **Normal weight (ml/min)** | **Obese (ml/min)** | **Normal weight (µl/min)** | **Obese (µl/min)** |
| Number | 20 | 20 | 20 | 20 |
| Mean | 0.56 | 0.68 | 0.6 | 0.8 |
| SD | 0.21 | 0.29 | 0.1 | 0.2 |
| SEM | 0.05 | 0.06 | 0.02 | 0.03 |
| p value | 0.146 | | 0.0001 | |

UWMS, unstimulated whole mouth saliva; GCF, gingival crevicular fluid; SD, standard deviation; SEM, standard error of mean.

### 4.1.3.2 Comparison of obesity biomarkers between normal weight and obese groups

Adiponectin was significantly reduced in GCF (p=0.025) and serum (p=0.032) of the obese subjects compared to normal weight, whilst levels were similar in UWMS (p=0.223). Leptin was significantly reduced in UWMS of the obese group (p=0.00004); however, in GCF and serum levels were comparable in both groups (p=0.103, p=0.977), respectively. No differences in resistin levels were observed between obese and normal weight groups in UWMS, GCF and serum (p=0.234, p=0.297, p=0.075), respectively (Figure 4.2, Table 4.4).

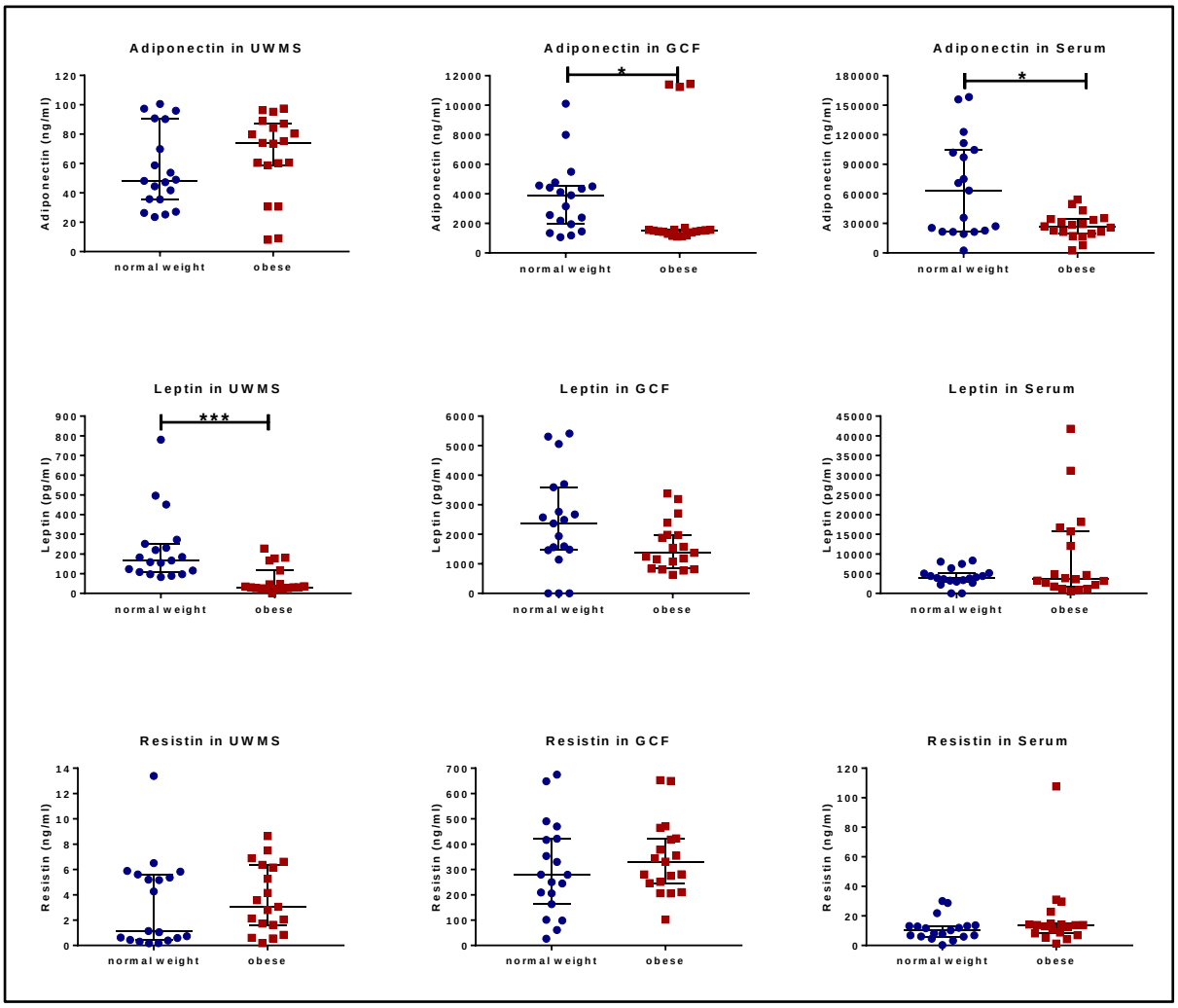



**Figure 4.2** Scatterplots showing the concentrations of obesity biomarkers in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight subjects in control cohort (median with 95%CI); *, significant difference ($p<0.05$); ***, significant difference ($p<0.001$).

### 4.1.3.3 Comparison of tissue remodelling biomarkers between normal weight and obese groups

MMPs such as MMP8 and MMP9 are crucial for PDL remodelling in both physiological and pathological conditions, with their actions regulated by inhibitors, such as TIMP1.

The levels of MMP8 were comparable in UWMS and GCF ($p=0.234$, $p=0.284$), respectively, and significantly higher in serum of the obese group compared to normal weight ($p=0.001$). MMP9 showed comparable levels in UWMS, GCF and serum ($p=0.977$, $p=0.138$, $p=0.686$), respectively. The levels of the inhibitor, TIMP1, were comparable in UWMS and GCF ($p=0.644$, $P=0.863$); however, in serum the levels were significantly higher in the obese group ($p=0.0138$) (Figure 4.3, Table 4.4).

Expressing the ratio of MMPs to TIMP levels provides a measure of the balance between the ECM damage done by MMPs and the inhibition of these effects by TIMPs during remodelling. UWMS, GCF and serum showed comparable levels of MMP8/TIMP1 ratio (p=0.191, p=0.751, p=0.470) and MMP9/TIMP1 ratio (p=0.708, p=0.234, p=0.370), respectively in both obese and normal weight groups (Figure 4.4, Table 4.4).

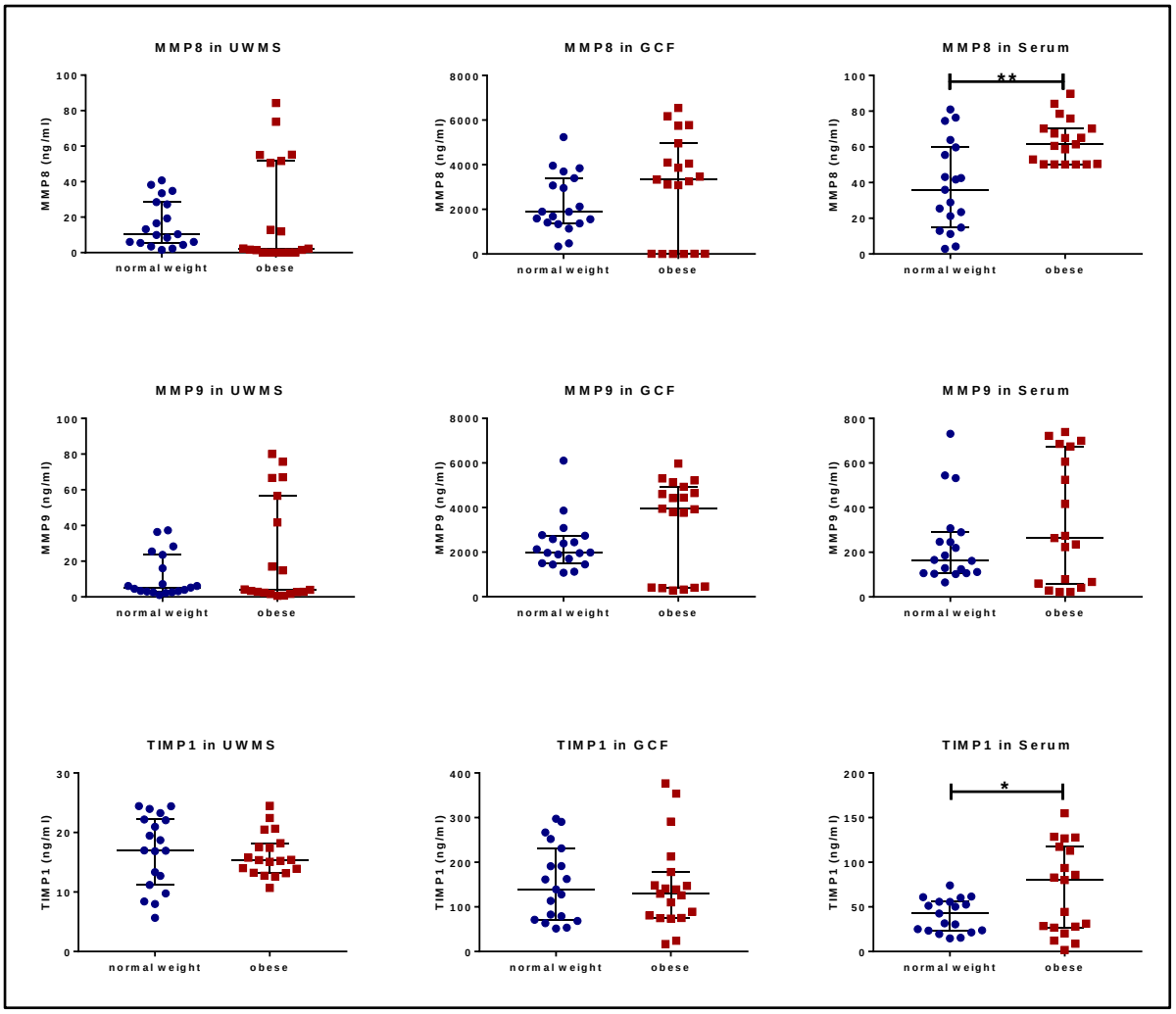



**Figure 4.3** Scatterplots showing the concentrations of tissue remodelling biomarkers in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight subjects in control cohort (median with 95%CI); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1 *, significant difference ($p<0.05$); **, significant difference ($p<0.01$).

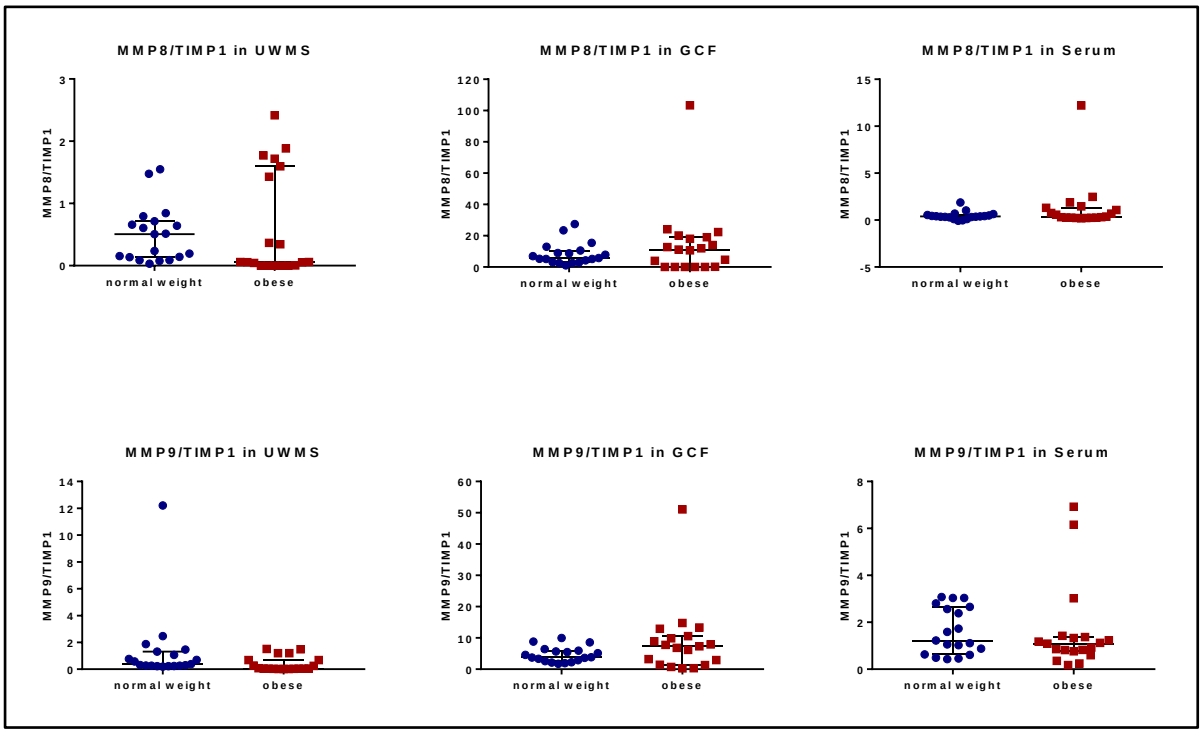



**Figure 4.4** Scatterplots showing the levels of MMPs/inhibitor ratios in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight subjects in control cohort (median with 95%CI). MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1.

In addition, zymography was used to detect the gelatinases (MMP2 and MMP9) in UWMS of 20 obese and 20 normal weight adults. Bands were seen as bright bands within the stained gel at 72 kDa for MMP2, 92 kDa and 120 kDa for MMP9. The results showed the highest band intensity at 120 kDa, then 92 kDa and the least at 72 kDa in both obese and normal weight groups. However, Kruskal-Wallis test showed no statistical difference between the three detected bands in both normal weight and obese groups (p=0.984, p=0.778), respectively (Table 4.3, Figure 4.5).

**Table 4.3** Mean of gelatinase bands intensity in UWMS of 20 obese and 20 normal weight subjects in control cohort.

| | **Normal weight** | | | **Obese** | | |
|---|---|---|---|---|---|---|
| MW | **120 kDa** | **92 kDa** | **72kDa** | **120 kDa** | **92 kDa** | **72kDa** |
| mean | 12149302 | 2541689 | 710033.3 | 16888026 | 4578699 | 3615717 |
| SD | 21753688 | 3481653 | 631489.3 | 42732128 | 8348219 | 10095393 |
| SEM | 4864273 | 778521.2 | 141205.3 | 9555194 | 1866719 | 2257399 |
| P value | 0.984 | | | 0.778 | | |

MW, molecular weight of gelatinase; SD, standard deviation; SEM, standard error of mean.

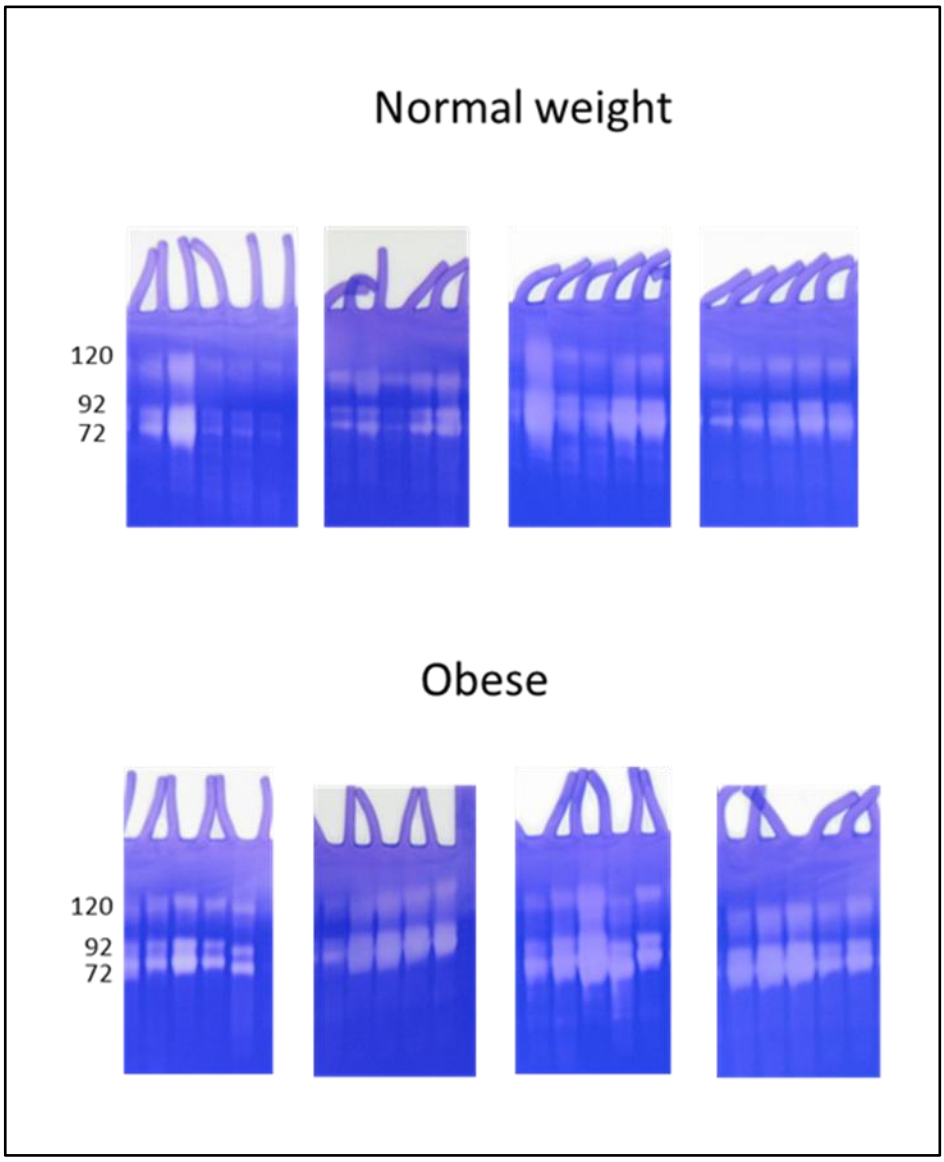



**Figure 4.5** Bands intensity of gelatinase in unstimulated whole mouth saliva of 20 obese and 20 normal weight subjects in control cohort detected by zymography using the ChemiDoc Imaging system (BioRad) laboratories ltd, Herts, UK.

### 4.1.3.4 Comparison of bone remodelling biomarker between normal weight and obese groups

The wide range of pro-inflammatory mediators produced by adipocytes of obese subjects stimulates the RANKL/RANK pathway, which in turn enhances osteoclastogensis and bone resorption.

The levels of RANKL were significantly higher in GCF (p=0.001) and serum (p=0.007) and significantly lower in UWMS (p=0.0001) of the obese group compared to normal weight (Figure 4.6, Table 4.4).

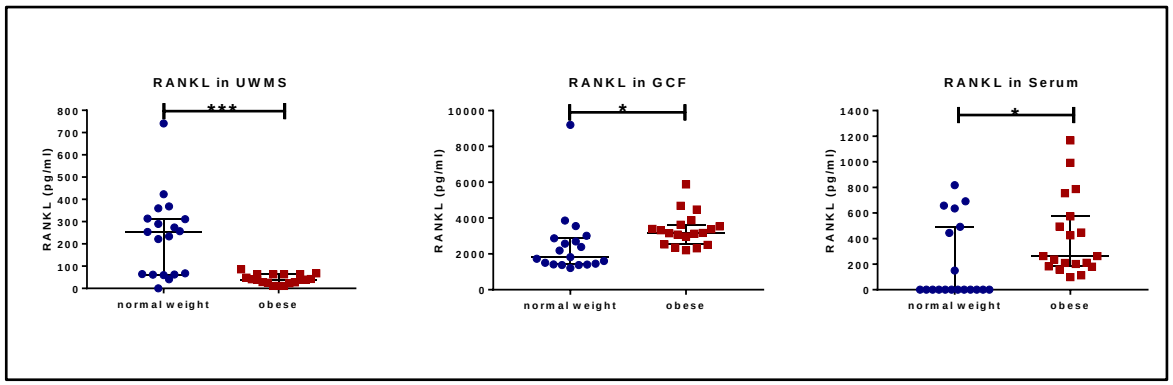



**Figure 4.6** Scatterplots showing the concentrations of bone remodelling biomarker in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight adults in control cohort (median with 95%CI); RANKL, receptor activator of nuclear factor kappa-B ligand; **, significant difference ($p<0.01$); ***, significant difference ($p<0.001$).

### 4.1.3.5 Comparison of inflammation biomarkers between normal weight and obese groups

Diverse inflammatory mediators are released by adipocytes, provoking a systemic inflammatory state that may negatively affect the general health status. Certain biomarkers such as CRP and MPO were used for the diagnosis and prediction of acute inflammation and tissue damage.

The levels of MPO were higher in obese group compared to normal weight in UWMS, GCF and serum ($p=0.002$, $p=0.0001$, $p=0.006$), respectively. However, the levels of CRP were significantly higher only in serum of obese group ($p=0.003$) and comparable in UWMS and GCF ($p=0.103$, $p=0.091$), respectively of the two groups (Figure 4.7, Table 4.4).

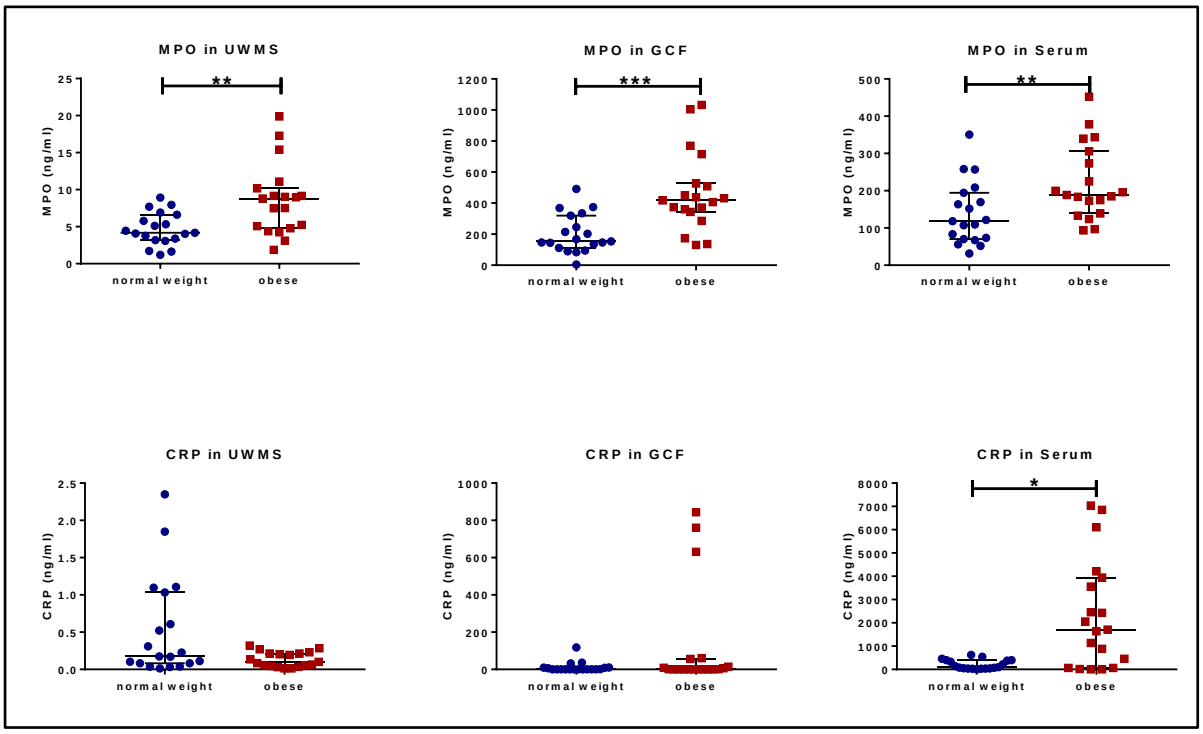



**Figure 4.7** Scatterplots showing the concentrations of inflammation biomarkers in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight subjects in control cohort (median with 95%CI); MPO, myeloperoxidase; CRP, C reactive protein; *, significant difference ($p<0.05$); **, significant difference ($p<0.01$); ***, significant difference ($p<0.001$).

### 4.1.3.6 Comparison of biomarkers between saliva, GCF and serum

The levels of the selected biomarkers were varied in different bio-fluids; the highest levels of some biomarkers were observed in serum followed by GCF and the least in UWMS such as adiponectin, leptin and CRP. However, the highest levels of the other biomarkers were observed in GCF followed by serum and then UWMS such as resistin, MMP8, MMP9, TIMP1, MPO and RANKL. These sequences were almost the same in both normal weight and obese groups (Table 4.4).

**Table 4.4** Mean and standard deviations of biomarkers' levels in UWMS, GCF and serum of 19 normal weight and 19 obese subjects without orthodontic treatment.

| Biomarkers | Sample | Normal weight | | Obese | |
|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD |
| Adiponectin (ng/ml) | UWMS | 55.86 | 26.81 | 65.85 | 27.80 |
| | GCF | 3763.81 | 2343.09 | 2980.59 | 3731.01 |
| | serum | 66255.87 | 49389.97 | 27428.94 | 12973.52 |
| Leptin (pg/ml) | UWMS | 224.49 | 176.27 | 67.09 | 68.82 |
| | GCF | 2374.33 | 1667.91 | 1606.38 | 823.15 |
| | serum | 4172.15 | 2289.91 | 8913.44 | 11357.47 |
| Resistin (ng/ml) | UWMS | 3.20 | 3.59 | 3.72 | 2.67 |
| | GCF | 301.30 | 184.55 | 344.41 | 144.83 |
| | serum | 9.91 | 9.85 | 18.28 | 22.97 |
| MMP8 (ng/ml) | UWMS | 16.30 | 13.30 | 21.32 | 29.37 |
| | GCF | 2261.78 | 1308.11 | 3026.44 | 2344.71 |
| | serum | 37.11 | 26.05 | 63.22 | 12.50 |
| MMP9 (ng/ml) | UWMS | 11.49 | 12.27 | 23.52 | 29.91 |
| | GCF | 2326.18 | 1151.66 | 3282.47 | 2100.75 |
| | serum | 235.81 | 180.49 | 336.02 | 280.92 |
| TIMP1 (ng/ml) | UWMS | 16.81 | 6.13 | 16.23 | 3.65 |
| | GCF | 152.37 | 83.83 | 146.80 | 99.83 |
| | serum | 40.54 | 18.69 | 69.08 | 49.48 |
| MMP8/ TIMP1 | UWMS | 0.49 | 0.44 | 0.62 | 0.83 |
| | GCF | 8.42 | 6.90 | 14.56 | 22.49 |
| | serum | 0.46 | 0.42 | 1.32 | 2.64 |
| MMP9/ TIMP1 | UWMS | 0.16 | 0.14 | 0.41 | 0.53 |
| | GCF | 4.66 | 2.39 | 8.83 | 10.92 |
| | serum | 1.62 | 0.97 | 1.60 | 1.80 |
| MPO (ng/ml) | UWMS | 4.68 | 2.20 | 8.56 | 4.78 |
| | GCF | 201.51 | 124.10 | 467.10 | 254.00 |
| | serum | 139.12 | 84.45 | 221.29 | 100.55 |
| CRP (ng/ml) | UWMS | 0.52 | 0.67 | 0.13 | 0.10 |
| | GCF | 11.82 | 27.84 | 283.49 | 651.35 |
| | serum | 208.45 | 201.72 | 2348.35 | 2343.88 |
| RANKL (pg/ml) | UWMS | 231.71 | 179.96 | 41.81 | 21.57 |
| | GCF | 2485.61 | 1809.75 | 3347.43 | 911.43 |
| | serum | 204.51 | 302.52 | 408.27 | 312.59 |

SD: standard deviation; UWMS: unstimulated whole mouth saliva; GCF: gingival crevicular fluid; MPO, myeloperoxidase; CRP, C reactive protein; MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; RANKL, receptor activator of nuclear factor kappa-B ligand.

### 4.1.3.7 Comparison of albumin and sIgA in UWMS of normal weight and obese subjects by immunoblotting

Western immunoblotting was used to detect other biomarkers in 20 obese and 20 normal weight adults. Unfortunately, because of the limited amount of the collected GCF, these proteins were detected only in UWMS by Western blot and immunoblotting. Band intensity of albumin and sIgA in UWMS of 20 normal weight and 20 obese subjects were quantified by scanning image analysis as shown in Figure 4.8 and, Figure 4.9 respectively, with no statistical significant differences for both albumin (p=0.484), and sIgA (p=0.314) were observed between normal weight and obese groups.

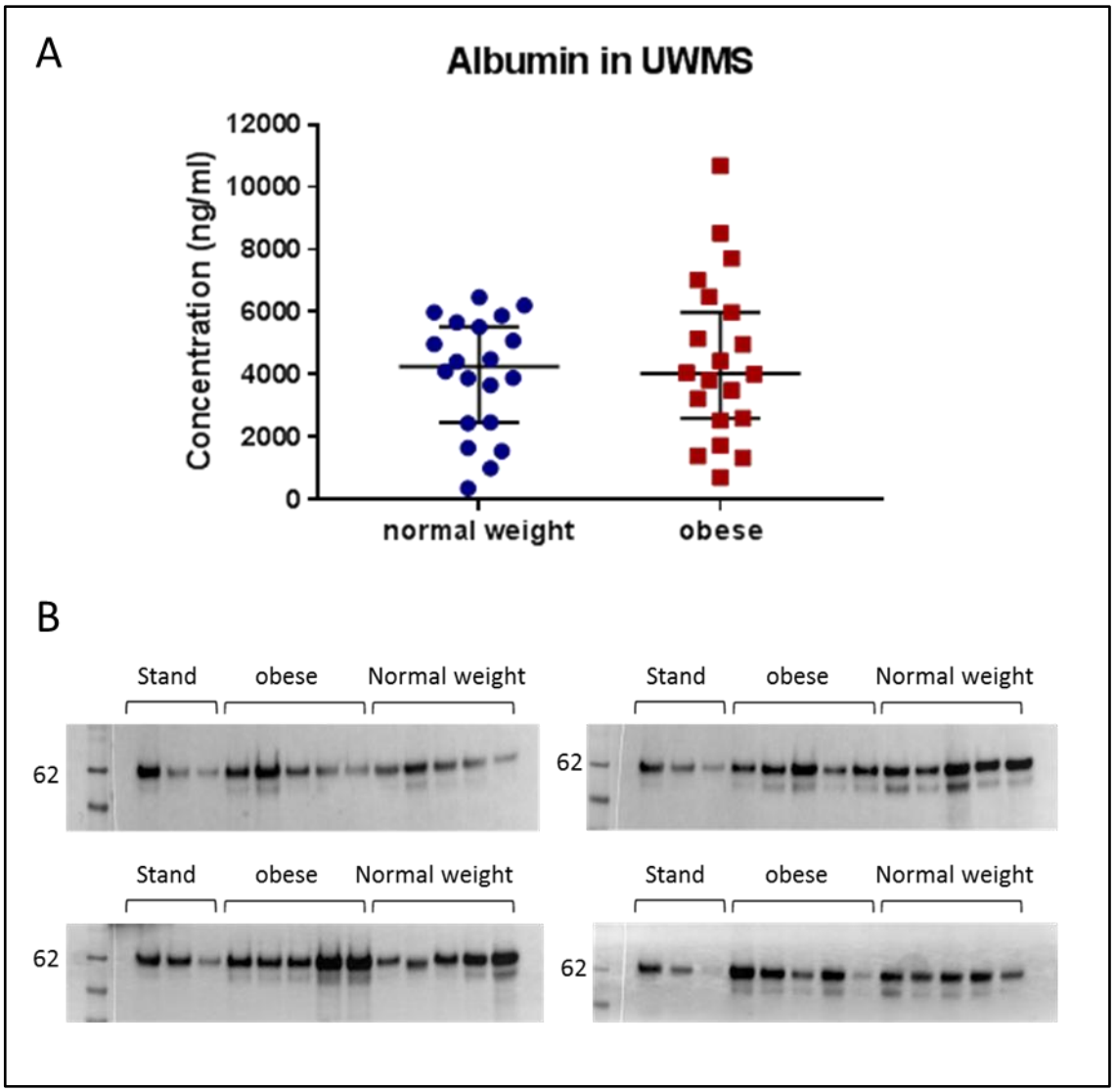



**Figure 4.8** Albumin in unstimulated whole mouth saliva (UWMS) of 20 obese and 20 normal weight` adults in control cohort. (A) Scatterplots (median with 95%CI), (B) Immunoblotting images. Stand: standard.

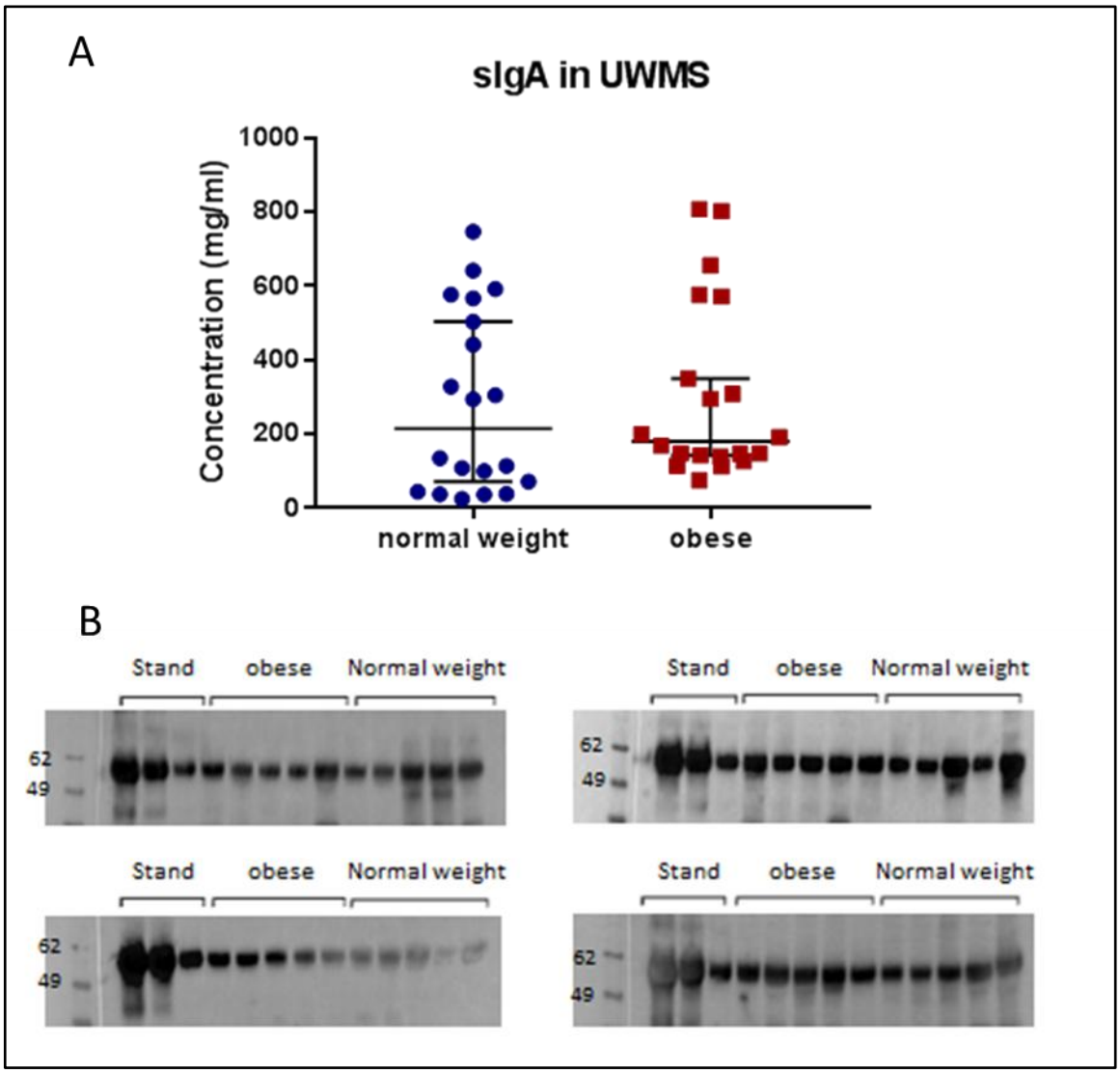



**Figure 4.9** Secretory IgA in unstimulated whole mouth saliva (UWMS) of 20 obese and 20 normal weight adults in control cohort. (A) Scatterplots (median with 95%CI), (B) Immunoblotting images, stand: standard.

# 4.2 Orthodontic treatment cohort

## 4.2.1 Introduction

When an orthodontist applies pressure and forces to a tooth, the patient will experience some local inflammation around the tooth, and this in turn increases capillary permeability in the paradental tissues and leads to the production of compounds that allow connective tissues and the surrounding bone to remodel (Storey, 1973). The compounds that are produced include cytokines and hormones, which are vital for repair (Teitelbaum, 2000, Sandy et al., 1993).

In those who are obese, it has been found that adipocytes and associated immune cells increase the production of pro-inflammatory proteins; such as leptin, resistin, TNF-α and IL-6, whilst at the same time, they cause a reduction in anti-inflammatory proteins, such as adiponectin, and this causes a degenerative low-grade inflammatory state (Fantuzzi, 2005, Nakamura et al., 2014), which can heighten the risk of obese individuals contracting periodontitis. Thus, it is argued that obesity can affect orthodontic treatment, namely pro-inflammatory changes in the PDL space, which can have a negative effect on the remodelling of tissue in the PDL.

It has been widely argued that venous blood is the best body fluid in terms of assessing the systemic process. Nevertheless, biochemical analysis of GCF is also an insightful method in terms of assessing the changes at one single site within a specific timeframe, in addition to determining the reaction of the dental and periodontal tissues to fixed orthodontic forces (Lee et al., 2004, Tuncer et al., 2005). It is likely that the same principle can also be applied to the use of whole saliva, and this is due to the fact that it is made up of the same components in GCF (Akalın et al., 2007).

The current literature reveals that obesity has an effect on the remodelling of bone metabolism, via various mechanisms, and these may have a significant effect on OTM (Cao, 2011). Conversely, forces applied by orthodontic appliance give rise to complicated mechanical and biological events such as matrix strain and fluid flow, cell strain, cell activation and differentiation, and remodelling which result in OTM (Henneman et al., 2008). Therefore, it can be argued both obesity and OTM pervert the standard physiological mechanisms of tissue and bone metabolism. Nevertheless, the potential changes of biomarkers during OTM in obese humans have not yet been characterised.

#### 4.2.1.1 Aims and objectives

The specific aims of this study were to investigate the effect of obesity on selected biomarkers during orthodontic treatment and to measure biomarker levels in saliva, GCF and serum.

To address these aims the following steps were undertaken:

1- Comparing the levels of biomarkers in obese and normal weight patients with fixed orthodontic appliances; and

2- Measuring the levels of the biomarkers in UWMS, GCF and serum.

The hypothesis was that obesity does not change the levels of selected biomarkers in patients undergoing fixed appliance orthodontic treatment.

### 4.2.2 Methods

The study was approved by the United Kingdom National Research Ethics Service, NRES Committee foundation (REC reference: 14/LO/0769, October 2014). Informed written consent was obtained from all participants.

#### 4.2.2.1 Participants and sampling

A total of 47 adult orthodontic patients (23 males and 24 females) under treatment with fixed orthodontic appliance (precoated 3M Victory 0.022-inch bracket, MBT prescription, 3M Unitek, Monrovia, USA) with 0.019 x 0.025-inch stainless steel rectangular archwires in the upper and lower arches were recruited as described in Section 2.4, number 2. Height and weight were measured for all participants to calculate BMI as shown in Section 2.2.1. According to the BMI 9 patients (4 males and 5 females) were classified as overweight (BMI = 25 - 30), and were excluded from the study. The sample therefore consisted of 19 normal weight (BMI= 18.5 - 25) (9 males and 10 females) with a mean age of 24.6 years (range 18-35 years) and 19 obese (BMI ≥30) (9 males and 10 females) with a mean age of 26.6 years (range 18-42 years) (Figure 4.10).

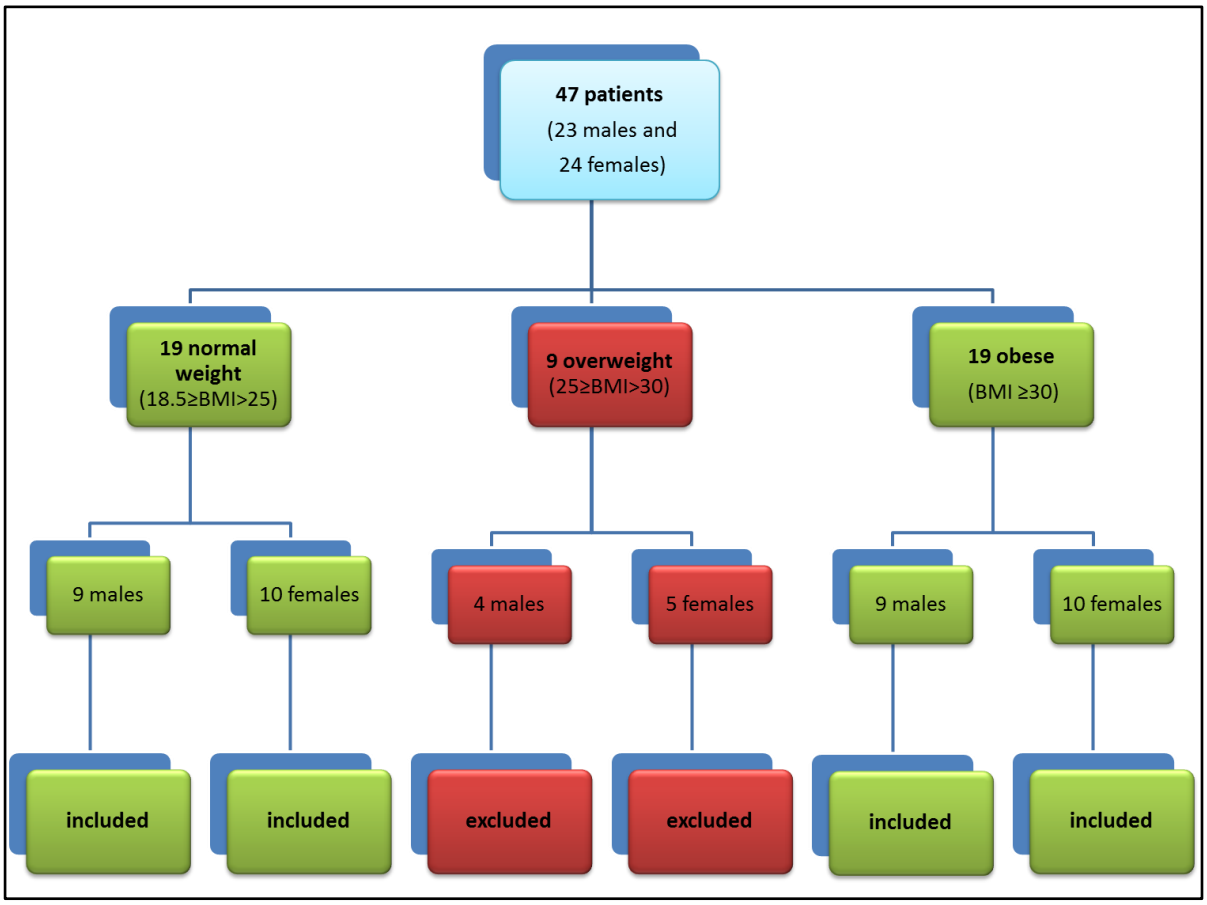



**Figure 4.10** Diagram for sample distribution of patients with orthodontic treatment.

For all participants, plaque levels and gingival health were measured as described in Section 2.3. UWMS, GCF and peripheral blood were collected as described in Section 2.4. UWMS and GCF flow rate were measured as mentioned in Section 2.6. The levels of the selected biomarkers were determined using a magnetic Luminex screening assay (R&D Systems, Bio-Techne; Abingdon, UK) as described in Section 2.13.

The study size and statistical analysis were the same as the control cohort mentioned in Sections 4.1.2.2 and 4.1.2.3, respectively with additional correlation tests to correlate the levels of biomarkers with the plaque and gingival indices scores.

### 4.2.3 Results

The clinical parameters of the subjects enrolled in the present study are shown in Table 4.5. Age did not significantly differ between the obese and normal weight groups ($p=0.338$). BMI values of the obese group were significantly higher than those of the normal weight group ($p<0.0001$).

**Table 4.5** Clinical characteristics of the patients with orthodontic treatment.

| Parameters | Normal weight | Obese |
|---|---|---|
| Number | 19 | 19 |
| Age (years): Mean (SD) | 24.6 (6.5) | 26.6 (6.2) |
| Gender: male / female | 9 / 10 | 9 / 10 |
| BMI ($kg/m^2$): Mean (SD) | 22.6 (1.6) | 32.4 (2.2)* |

SD: standard deviation, BMI: body mass index, *=significantly higher ($p<0.0001$) using independent t-test.

### 4.2.3.1 UWMS and GCF flow rate

Salivary and GCF flow rate can be affected with the presence of orthodontic appliance as well as the inflammatory reactions take place during OTM.

Independent t test showed that in both obese and normal weight groups, UWMS and GCF flow rates were comparable (p=0.73, p=0.06), respectively (Table 4.6).

**Table 4.6** UWMS and GCF flow rate of patients with orthodontic treatment.

| | UWMS flow rate | | GCF flow rate | |
|---|---|---|---|---|
| | **normal weight (ml/min)** | **Obese (ml/min)** | **normal weight (µl/min)** | **Obese (µl/min)** |
| Number | 19 | 19 | 19 | 19 |
| Mean | 0.68 | 0.65 | 0.86 | 0.89 |
| SD | 0.31 | 0.26 | 0.17 | 0.21 |
| SEM | 0.07 | 0.06 | 0.04 | 0.05 |
| p value | 0.73 | | 0.06 | |

UWMS, unstimulated whole mouth saliva; GCF, gingival crevicular fluid; SD, standard deviation; SEM, standard error of mean.

### 4.2.3.2 Comparison of obesity biomarkers between normal weight and obese groups

The levels of adiponectin were comparable in UWMS, GCF and serum (p=0.795, p=0.258, p=0.130) respectively. Leptin levels in UWMS were also comparable in both obese and normal weight groups (p=0.116). However, it was significantly higher in GCF (p=0.009) and serum (p=0.001) in the obese group in comparison to those of normal weight. No differences in resistin levels were observed between the obese and normal weight groups in UWMS, GCF and serum (p=0.246, p=0.435, p=0.708), respectively (Figure 4.11, Table 4.7).

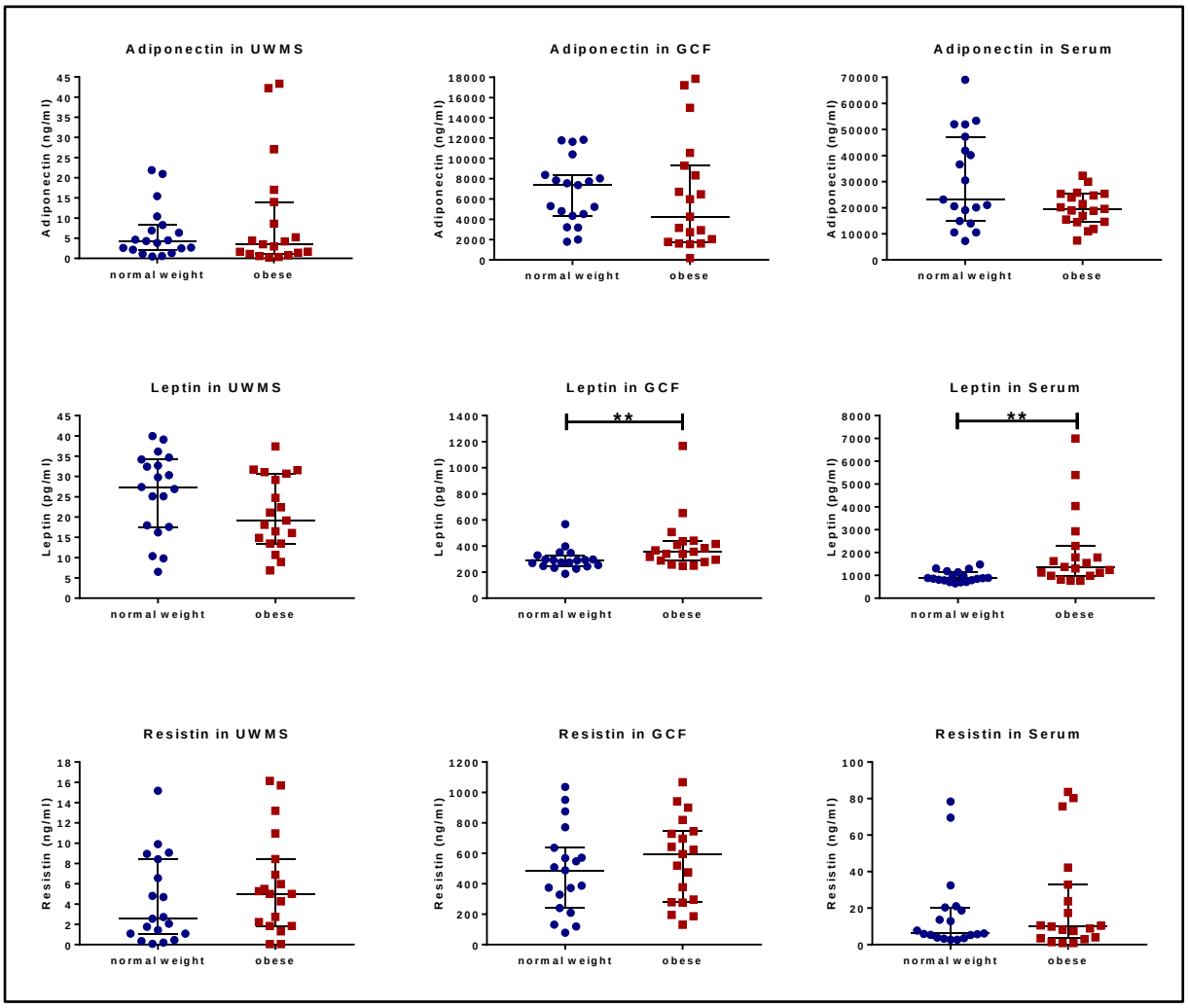



**Figure 4.11** Scatterplots showing the concentrations of obesity biomarkers in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight patients in orthodontic treatment cohort (median with 95%CI); **, significant difference ($p<0.01$).

### 4.2.3.3 Comparison of tissue remodelling biomarkers between normal weight and obese groups

OTM requires extensive remodelling of the periodontium. MMPs such as MMP8 and MMP9 degrade the ECM during remodelling, while their activity is regulated by the tissue inhibitors such as TIMP1.

The levels of MMP8 were comparable in UWMS, GCF and serum ($p=0.452$, $p=0.223$ and $p=0.840$), respectively. MMP9 showed comparable levels in UWMS ($p=0.885$) whereas its level was significantly higher in GCF ($p=0.020$) and serum ($p=0.034$) of the obese group in comparison to normal weight. The levels of the inhibitor, TIMP1,

were comparable in UWMS and GCF (p=0.085, p=1.000) respectively; however, in serum it was significantly higher in obese group (p=0.002) (Figure 4.12, Table 4.7).

Since the effect of MMPs is controlled by the inhibitor TIMPs, therefore measuring the MMP/TIMP ratio is more objective. No differences were observed in MMP8/TIMP1 and MMP9/TIMP1 ratios between the obese and normal weight groups in UWMS, GCF and serum (MMP8/TIMP1, p=0.751, p=0.603, p=0.212, MMP9/TIMP1, p=1.000, p=0.435, p=0.644), respectively (Figure 4.13, Table 4.7).

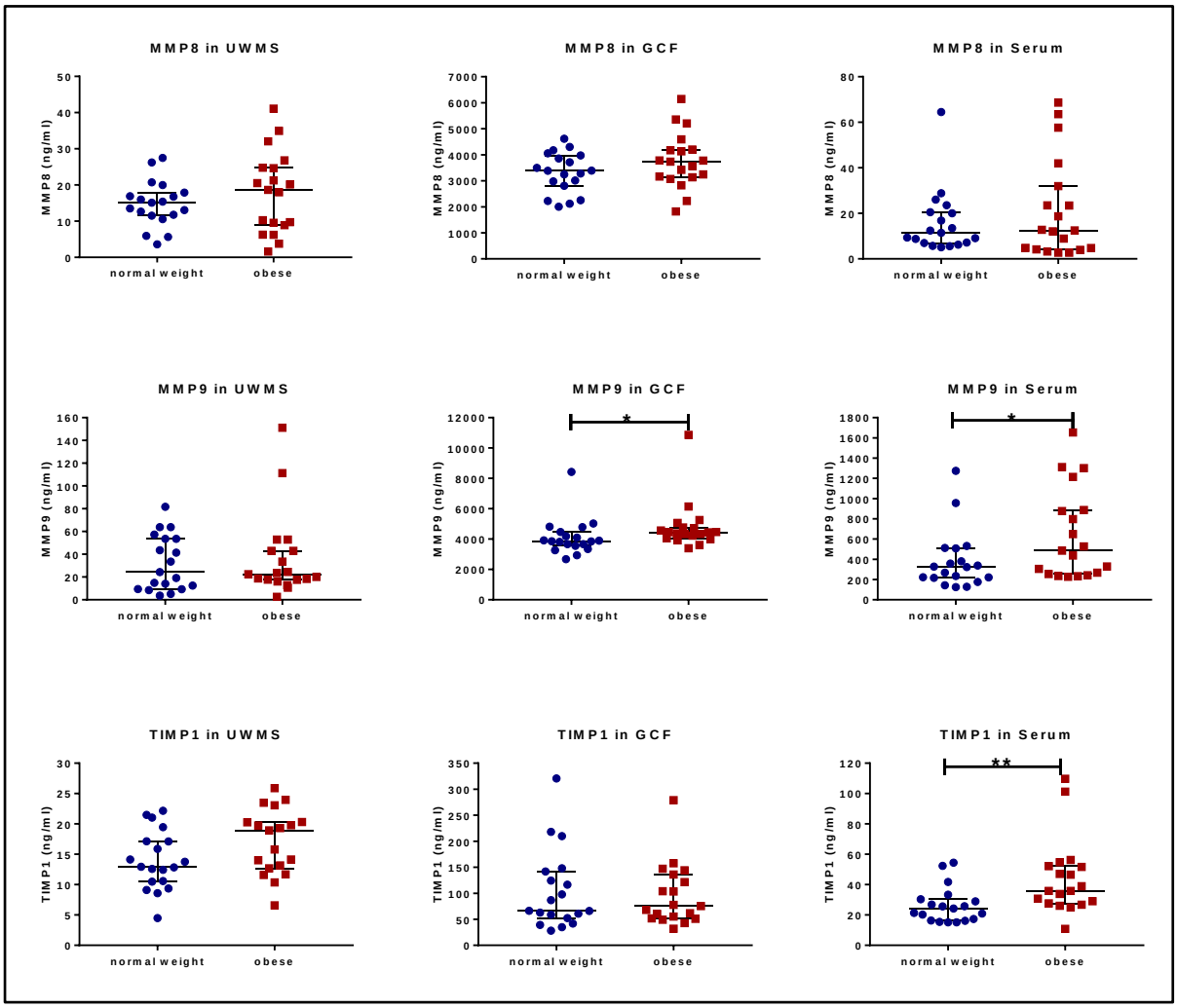



**Figure 4.12** Scatterplots showing the concentrations of tissue remodelling biomarkers in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight patients in orthodontic treatment cohort (median with 95%CI); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; *: significant difference (p<0.05); **: significant difference (p<0.01).

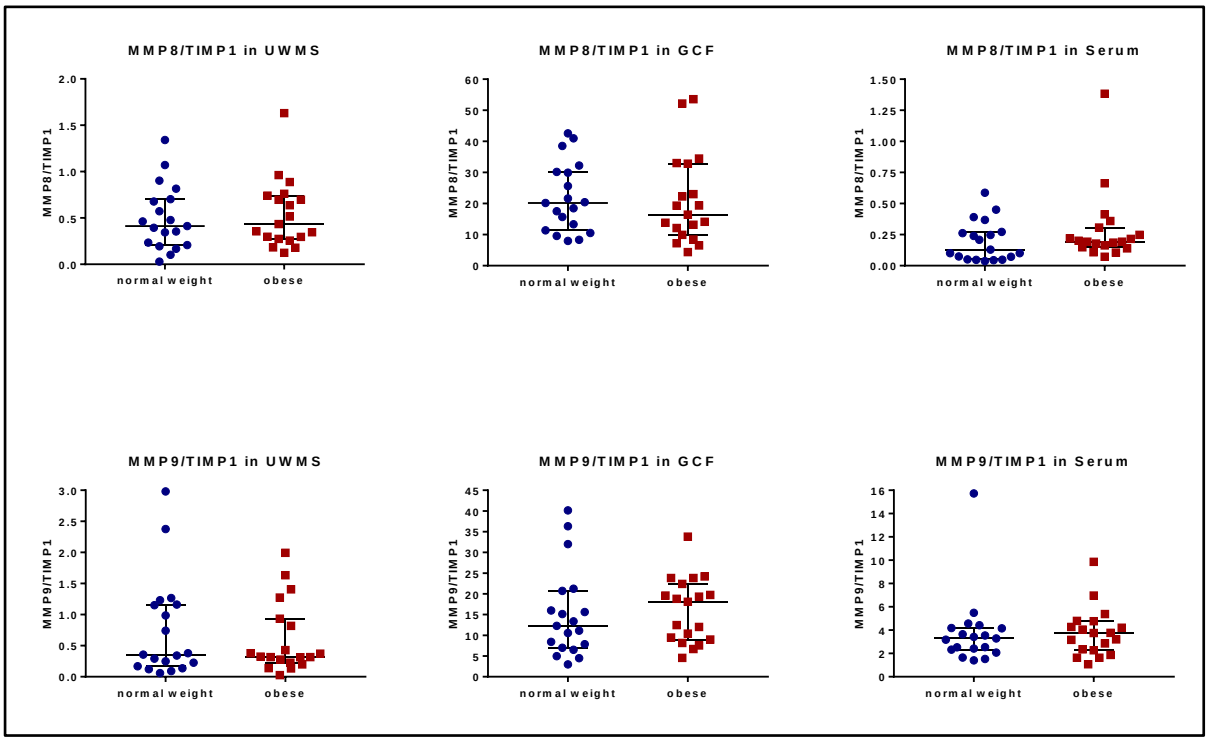



**Figure 4.13** Scatterplots showing the levels of MMPs/inhibitor ratios in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight patients in orthodontic treatment cohort (median with 95%CI); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1.

### 4.2.3.4 Comparison of bone remodelling biomarker between normal weight and obese groups

Since the RANKL/RANK pathway is a key for osteoclastogensis and bone resorption, it is important to consider levels of RANKL during OTM.

The levels of RANKL were comparable in UWMS and GCF (p=0.080, p=0.624) in both normal weight and obese groups (Figure 4.14, Table 4.7). However, in serum, RANKL levels were below the detection limit in all samples of both groups.

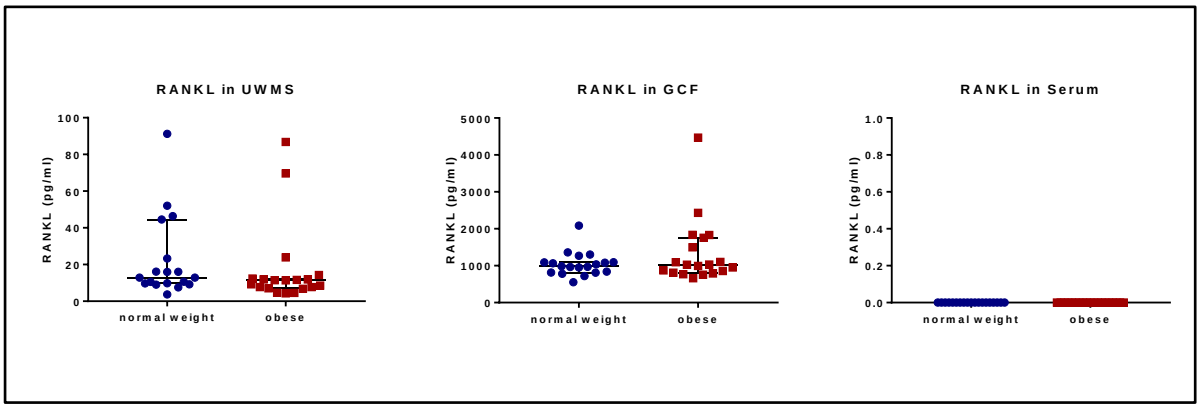



**Figure 4.14** Scatterplots showing the concentrations of bone remodelling biomarker in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight patients in orthodontic treatment cohort (median with 95%CI); RANKL, receptor activator of nuclear factor kappa-B ligand.

### 4.2.3.5 Comparison of inflammation biomarkers between normal weight and obese groups

Forces during orthodontic treatment create acute inflammation in the surrounding supporting structure. Certain biomarkers such as CRP and MPO were used for the diagnosis and prediction of acute inflammation and tissue damage in many systemic diseases. The levels of MPO and CRP were comparable in both obese and normal weight groups in UWMS, GCF and serum (MPO: p=0.061, p=0.773, p=0.773; CRP: p=0.191, p=0.075, p=0.109), respectively (Figure 4.15, Table 4.7).

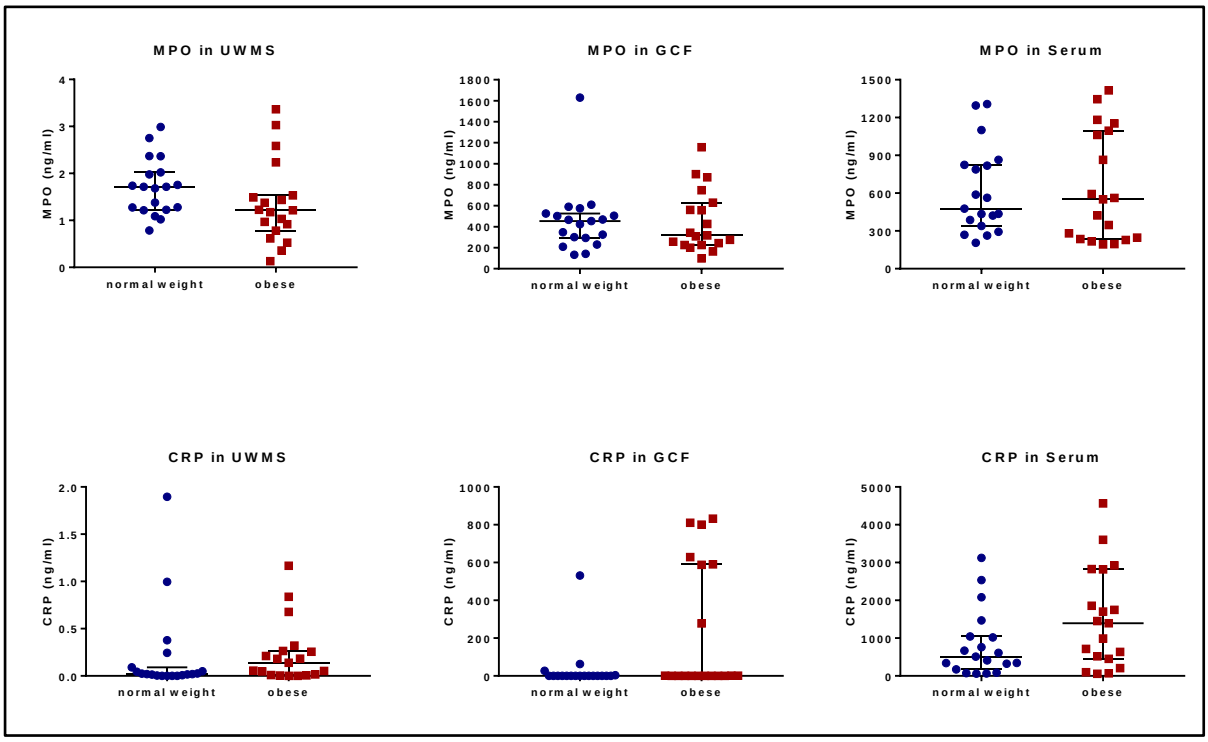



**Figure 4.15** Scatterplots showing the concentrations of inflammation biomarkers in unstimulated whole mouth saliva (UWMS), gingival crevicular fluid (GCF) and serum of 19 obese and 19 normal weight patients in orthodontic treatment cohort (median with 95%CI); MPO, myeloperoxidase; CRP, C reactive protein.

### 4.2.3.6 Comparison of biomarkers between saliva, GCF and serum

The levels of the selected biomarkers were varied in different bio-fluids, the highest levels of some biomarkers were observed in serum followed by GCF and the least in UWMS such as adiponectin, leptin, MPO and CRP. However, the highest levels of the other biomarkers were observed in GCF followed by serum and then UWMS such as resistin, MMP8, MMP9 and TIMP1. RANKL registered the highest level in GCF followed by UWMS but could not be detected in serum in both normal weight and obese groups. These sequences were almost the same in both normal weight and obese groups (Table 4.7)

**Table 4.7** Mean and standard deviations of biomarkers' levels in UWMS, GCF and serum of 19 normal weight and 19 obese patients with orthodontic treatment.

| Biomarkers | Sample | Normal weight | | Obese | |
|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD |
| Adiponectin (ng/ml) | UWMS | 6.36 | 6.48 | 9.49 | 13.61 |
| | GCF | 6683.80 | 3224.31 | 6278.34 | 5480.16 |
| | serum | 30724.40 | 17961.21 | 19878.73 | 6572.60 |
| Leptin (pg/ml) | UWMS | 25.90 | 10.14 | 20.94 | 8.92 |
| | GCF | 298.30 | 81.69 | 407.77 | 209.19 |
| | serum | 936.33 | 232.89 | 2044.38 | 1691.96 |
| Resistin (ng/ml) | UWMS | 4.29 | 4.26 | 4.99 | 4.80 |
| | GCF | 484.14 | 280.57 | 552.56 | 279.73 |
| | serum | 16.80 | 21.73 | 22.35 | 27.85 |
| MMP8 (ng/ml) | UWMS | 14.78 | 6.27 | 17.85 | 11.15 |
| | GCF | 3310.32 | 775.50 | 3768.19 | 1057.75 |
| | serum | 15.89 | 13.92 | 21.16 | 21.66 |
| MMP9 (ng/ml) | UWMS | 32.27 | 24.27 | 36.45 | 36.73 |
| | GCF | 4117.81 | 1209.17 | 4787.23 | 1590.90 |
| | serum | 381.29 | 291.63 | 643.95 | 448.42 |
| TIMP1 (ng/ml) | UWMS | 13.98 | 4.84 | 26.41 | 5.34 |
| | GCF | 103.98 | 76.43 | 95.66 | 59.34 |
| | serum | 26.41 | 11.85 | 44.21 | 24.73 |
| MMP8/ TIMP1 | UWMS | 0.54 | 0.36 | 0.50 | 0.34 |
| | GCF | 20.86 | 14.00 | 21.84 | 10.86 |
| | serum | 0.29 | 0.29 | 0.20 | 0.16 |
| MMP9/ TIMP1 | UWMS | 0.75 | 0.79 | 0.61 | 0.56 |
| | GCF | 15.10 | 10.46 | 15.99 | 7.61 |
| | serum | 3.79 | 3.01 | 3.78 | 2.03 |
| MPO (ng/ml) | UWMS | 1.70 | 0.60 | 1.37 | 0.87 |
| | GCF | 459.47 | 319.76 | 447.57 | 293.67 |
| | serum | 614.62 | 343.40 | 641.62 | 436.81 |
| CRP (ng/ml) | UWMS | 0.20 | 0.47 | 0.23 | 0.32 |
| | GCF | 33.02 | 121.61 | 249.10 | 360.31 |
| | serum | 825.95 | 883.40 | 150.69 | 1312.66 |
| RANKL (pg/ml) | UWMS | 37.34 | 69.05 | 17.13 | 22.17 |
| | GCF | 1040.51 | 324.95 | 1343.97 | 894.56 |
| | serum | 0 | 0 | 0 | 0 |

SD, standard deviation; UWMS: unstimulated whole mouth saliva; GCF: gingival crevicular fluid; MPO, myeloperoxidase; CRP, C reactive protein; MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; RANKL, receptor activator of nuclear factor kappa-B ligand.

### 4.2.3.6 Correlation of biomarkers with periodontal health

Certain biomarkers are produced as a response to local stimuli or inflammation, therefore bacterial plaque and inflamed gingivae can potentially influence the levels of these biomarkers.

Both normal weight and obese patients showed comparable values of plaque and gingival scores (p=0.662, p=0.302), respectively by independent t-test (Table 4.18).

**Table 4.8** Plaque and gingival indices values for normal weight and obese patients with orthodontic treatment.

| | **Plaque index values** | | **Gingival index values** | |
|---|---|---|---|---|
| | **normal weight** | **Obese** | **normal weight** | **Obese** |
| Number | 19 | 19 | 19 | 19 |
| Mean | 1.26 | 1.33 | 1.94 | 2.04 |
| SD | 0.42 | 0.50 | 0.29 | 0.28 |
| SEM | 0.10 | 0.11 | 0.07 | 0.06 |
| p value | 0.662 | | 0.302 | |

SD, standard deviation; SEM, standard error of mean.

In the normal weight group a correlation was observed only between the levels of MMP9 and plaque index in UWMS (r=0.51, p=0.027). In the obese group, the correlations were found only between the levels of MPO in UWMS with plaque index (r=0.583, p=0.009) and with gingival index (r=0.463, p=0.046) between the levels of CRP in UWMS and gingival index (r=0.461, p=0.047) (Table 4.9).

**Table 4.9** Correlation of analytes in UWMS, GCF and serum with plaque index (PI) and gingival index (GI) in the obese and normal weight patients with orthodontic treatment.

| Biomarkers | Samples | Normal weight | | | | Obese | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Plaque index | | Gingival index | | Plaque index | | Gingival index | |
| | | r | P value | r | P value | r | P value | r | P value |
| adiponectin | UWMS | 0.02 | 0.94 | 0.12 | 0.61 | 0.07 | 0.79 | 0.37 | 0.13 |
| | GCF | 0.09 | 0.73 | -0.09 | 0.72 | 0.02 | 0.95 | -0.15 | 0.53 |
| | serum | -0.24 | 0.32 | -0.23 | 0.35 | -0.04 | 0.88 | 0.15 | 0.55 |
| leptin | UWMS | 0.17 | 0.48 | 0.07 | 0.79 | 0.00 | 1.00 | -0.40 | 0.09 |
| | GCF | -0.30 | 0.21 | -0.24 | 0.33 | 0.09 | 0.73 | -0.14 | 0.57 |
| | serum | -0.17 | 0.49 | -0.29 | 0.24 | 0.10 | 0.67 | 0.14 | 0.56 |
| resistin | UWMS | 0.10 | 0.67 | 0.11 | 0.65 | 0.06 | 0.80 | 0.34 | 0.16 |
| | GCF | 0.18 | 0.45 | 0.11 | 0.67 | -0.25 | 0.31 | 0.00 | 0.99 |
| | serum | 0.09 | 0.72 | -0.30 | 0.21 | 0.23 | 0.34 | 0.07 | 0.78 |
| MMP8 | UWMS | -0.14 | 0.56 | 0.56 | 1.00 | 0.24 | 0.33 | 0.09 | 0.72 |
| | GCF | -0.22 | 0.37 | -0.07 | 0.79 | -0.43 | 0.07 | -0.45 | 0.05 |
| | serum | 0.03 | 0.89 | -0.27 | 0.27 | 0.20 | 0.42 | 0.05 | 0.85 |
| MMP9 | UWMS | **0.51*** | 0.03 | 0.42 | 0.07 | 0.09 | 0.71 | 0.34 | 0.16 |
| | GCF | -0.33 | 0.17 | -0.30 | 0.21 | -0.06 | 0.80 | -0.22 | 0.37 |
| | serum | 0.02 | 0.94 | -0.30 | 0.21 | 0.03 | 0.90 | 0.08 | 0.76 |
| TIMP1 | UWMS | -0.36 | 0.13 | -0.18 | 0.47 | 0.10 | 0.67 | -0.12 | 0.62 |
| | GCF | 0.43 | 0.07 | 0.00 | 1.00 | -0.06 | 0.82 | 0.02 | 0.95 |
| | serum | 0.07 | 0.78 | -0.17 | 0.49 | -0.04 | 0.86 | 0.07 | 0.78 |
| MPO | UWMS | 0.16 | 0.51 | 0.05 | 0.85 | **0.58**** | 0.01 | **0.46*** | 0.05 |
| | GCF | -0.39 | 0.10 | -0.39 | 0.10 | -0.04 | 0.89 | -0.31 | 0.20 |
| | serum | 0.28 | 0.25 | 0.00 | 0.99 | 0.16 | 0.52 | -0.02 | 0.95 |
| CRP | UWMS | 0.08 | 0.75 | 0.02 | 0.94 | 0.27 | 0.27 | **0.46*** | 0.05 |
| | GCF | 0.12 | 0.64 | 0.05 | 0.83 | 0.31 | 0.20 | 0.24 | 0.33 |
| | serum | -0.28 | 0.24 | -0.03 | 0.91 | -0.23 | 0.34 | 0.02 | 0.94 |
| RANKL | UWMS | 0.09 | 0.70 | -0.12 | 0.61 | 0.15 | 0.53 | -0.01 | 0.96 |
| | GCF | -24.00 | 0.30 | -0.27 | 0.26 | 0.09 | 0.73 | -0.14 | 0.56 |
| | serum | a | a | a | a | a | a | a | a |

MPO, myeloperoxidase; CRP, C reactive protein; MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; RANKL, receptor activator of nuclear factor kappa-B ligand; a: cannot be measured because all values are zero, *,correlation is significant at the 0.05 level (2-tailed); **, correlation is significant at the 0.01 level (2-tailed).

## 4.3 Discussion

The main aim of this study was to investigate the effects of obesity on the levels of biomarkers in different bio-fluids with and without fixed-appliance orthodontic treatment.

Both the chronic inflammation associated with obesity and the acute inflammation resulting from applied orthodontic forces produce a wide range of pro- and anti-inflammatory mediators. Therefore, we characterised the biochemical changes resulting from obesity alone by comparing the levels of selected biomarkers between normal weight and obese adults without orthodontic treatment (control cohort). We then characterised the same biomarkers in normal weight and obese adult patients undergoing fixed appliance orthodontic treatment with 0.019 x 0.025-inch stainless steel archwires in the upper and lower arches (orthodontic treatment cohort) to detect if obesity can modify the inflammatory status during orthodontic treatment. Additionally, levels of these biomarkers in different bio-fluids were characterised to investigate the local and/or systemic changes.

In the design of this study, the influence of age and sex were curtailed through the selection of volunteers with a minimum age of 18 years and a maximum age of 45 years with a matched age and gender, the rationale behind this is that people over 45 years are more likely to have artificial prosthesis and/or periodontal diseases that doesn't fall into the criteria of the control cohort, and less likely to undergo orthodontic treatment which doesn't fit the criteria of the orthodontic treatment cohort.

One of the factors affecting the composition of saliva and GCF is the flow rate. In this study, GCF flow rate increased significantly with obesity in control cohort subjects who had no orthodontic appliances (Table 4.2). Since there were no clear signs of gingivitis and plaque accumulation in all participants, the main reason for higher GCF flow rate could be due to subclinical inflammatory effects of obesity on the periodontal tissues, which may affect blood vessel permeability and increase GCF flow rate in obese subjects. This has been confirmed by previous studies finding that the rate and composition of GCF can be altered by inflammation (Kavadia-Tsatala et al., 2001). In the orthodontic treatment cohort, a higher GCF flow rate was observed in both normal weight and obese cohorts in comparison to control without orthodontic treatment. Such findings could be because orthodontic force causes a distortion of the ECM of the PDL and the production of molecules; some of which are vasoactive. In addition, the stretching and compression of capillaries in the deeper periodontal tissues may alter GCF flow rate and composition.

However, no significant differences were observed between normal weight and obese groups under orthodontic treatment (Table 4.6) which can be explained by increased GCF flow rate in both groups in response to orthodontic force exceeding that due to obesity. Comparable UWMS flow rates were observed between normal weight and obese groups with and without orthodontic treatment, indicating that obesity did not significantly influence saliva secretion. These results are in agreement with the findings of previous studies measuring salivary flow rate in obese adults (FenolI-Palomares et al., 2004) and children (Pannunzio et al., 2010).

Defensive proteins involved in both the innate and acquired immune system such as immunoglobulins can be found in saliva. Secretory immunoglobulin A (sIgA) is an important immune regulator at mucosal surfaces, with a secretory component mediating anchoring of the polymeric molecule to epithelial surfaces via glycan residues (Phalipon et al., 2002). It is mainly produced by the minor salivary and parotid glands (Eliasson et al., 2006, Sonesson et al., 2011), it is highly concentrated on oral epithelial cells to prevent the entry, attachment, replication and colonization of pathological microorganisms, along with neutralizing toxins and viruses (Fabian et al., 2008, Gibbins et al., 2014). In addition, it down regulates the pro-inflammatory response (Mantis et al., 2011). An absence of sIgA therefore increase the likelihood both mucosal infections and those of the respiratory tract (Fábián et al., 2007). The control cohort of the present study showed comparable levels of sIgA in UWMS of both obese and normal weight groups, which may indicate that obesity does not obviously affect the mucosal humeral adaptive immunity in the sample of this study (Figure 4.9).

Given the presence of proteins normally associated with serum in saliva, it is evident that there is diffusion of these proteins from serum to saliva, probably via GCF. Therefore, this study also measured serum albumin in UWMS to test diffusion of protein from serum to saliva via GCF. Comparable levels of salivary albumin were observed in both obese and normal weight groups, indicating that diffusion of proteins is similar in both groups and is not affected by obesity (Figure 4.8).

The strengths of the present study include obesity was defined according to widely accepted and reliable measures, which makes misclassification unlikely. However, some potential limitations include the fact that height and weight measurements were not taken before orthodontic treatment and adiposity is not necessarily a static measure. In addition, only BMI was used to classify adiposity, which can limit in terms of overestimating body

fat in people with unusually large muscle mass, as is frequently the case for trained athletes or body builders. These limitations could have been reduced by adding estimates of adiposity (body adiposity index), fat distribution (waist-to-height ratio) and estimate overweight (skin fold), but could not be done so as to avoid embarrassment of the participants. In addition, the salivary flow rate and protein levels are affected by circadian rhythm, however it is not ethical to make pressure on the participants to arrange their appointments at the same time during the day, and therefore all samples were taken during the day in routine clinics between the hours 9:30 am to 3:30 pm. The other limitation was that sample collection for the control cohort without orthodontic treatment was performed in a sample collection room rather than a dental clinic that inhibits the proper plaque and gingival score measurement and was based mainly on a visual assessment.

### 4.3.1 Obesity-related biomarkers

A number of specific proteins are secreted mainly by adipocytes and called adipokines, including adiponectin, leptin and resistin. Similar to previous researches, the present study confirmed the fact that although adiponectin, leptin and resistin belong to the same family of adipokines which are mostly regulated during obesity, they behave differently. In the control cohort (without orthodontic treatment), the levels of adiponectin in GCF and serum were reduced in obese subjects. These results agree with another study, which demonstrated a reduced level of plasma adiponectin and the adiponectin receptors 1 and 2 in obese individuals (Kadowaki and Yamauchi, 2005); however, salivary adiponectin was not affected by obesity which can confirm our findings of pseudo adiponectin in saliva. These findings match data from other studies, where significant correlations between plasma adiponectin levels and BMI were reported (Ryo et al., 2004, Galler et al., 2007, Toda et al., 2007), along with irrelevant correlations between salivary adiponectin levels and BMI (Toda et al., 2007, Toda and Morimoto, 2008, Mamali et al., 2012, Nigro et al., 2015). Conversely, in the orthodontic treatment cohort the levels of adiponectin in UWMS, GCF and serum were comparable in both obese and normal weight groups that can be explained by the effects of orthodontic force which are more than obesity result in reduced levels of anti-inflammatory protein, adiponectin, in both normal weight and obese groups.

Another obesity biomarker is leptin, previous researches suggested that leptin stimulates pro-inflammatory cytokines production and phagocytosis by macrophages

during the host response to infection and inflammatory stimuli (Ahima and Flier, 2000). In the present study, the obese subjects without orthodontic treatment showed significantly lower levels of salivary leptin in comparison to normal weight that may point to the effect of obesity on leptin production by salivary glands. These explanations, based on the findings of a previous study, indicated that human salivary glands express leptin at both mRNA and protein levels (Randeva et al., 2003). Furthermore, this trend in saliva differs from those in GCF and serum may indicate that obesity affects not only the production of leptin by salivary gland but also the diffusion of leptin from serum to saliva by changing the basement membrane permeability. The findings of this study are further supported by other studies found a lower ratio of salivary/plasma leptin in obese subjects in comparison to lean subjects (Gröschl et al., 2001). In GCF and serum, comparable levels of leptin were observed in both normal weight and obese groups, which disagree with a previous study mentioned that the serum levels of leptin correlate with body weight and fat mass as a result of a greater amount of leptin being produced by adipocytes (Considine et al., 1996). The similar levels of circulating leptin that we identify here might be related to the relatively low BMI of the obese subjects participating in this study (mean ± SD; 32.2 ± 2.5). In GCF, the comparable levels of leptin could be correlated with healthy periodontal conditions of the participants in both groups. These explanations based on the results of a previous study undertaken by Karthikeyan and Pradeep to compare levels of leptin in the GCF of periodontal healthy and diseased conditions. They found that these levels decreased with periodontal disease (Karthikeyan and Pradeep, 2007b).

Conversely, subjects with orthodontic treatment showed that the levels of leptin increased in both GCF and serum of obese group. This could be explained by higher TNF-α, IL-1β and other cytokines during orthodontic treatment (Uematsu et al., 1996b, Perinetti et al., 2002) which consequently increase the production of leptin (Bornstein et al., 1998, Wallace et al., 2000). Furthermore, leptin has been reported to have similar structure and function of cytokines (Ahima and Flier, 2000) which are significantly increased with inflammatory conditions associated with obesity and OTM. Similarly, serum levels of leptin have been demonstrated to be increased with higher levels of cytokines such as cortisol, TNF-α, IL-6 (Bornstein et al., 1998, Wallace et al., 2000). In contrast to our findings, other authors reported reduced levels of leptin in GCF by orthodontic treatment (Dilsiz et al., 2010), these incomparable findings could be related to differences in BMI,

age and assay used for biochemical analyses. For instance Dilsiz and co-workers used ELISA assay to investigate leptin in GCF of normal weight teenagers.

Although resistin is considered to be an obesity-related biomarker, in this study, its highest levels were observed in GCF followed by serum then UWMS with no significant differences between the obese and normal weight groups in any of the three samples with and without orthodontic treatment. Similarly, a previous study conducted by Patel and Raju (2014) comparing resistin levels in GCF and serum from obese and normal individuals with and without periodontitis. They found that resistin levels were higher in GCF than serum, and elevated with periodontitis with no significant increase with obesity (Patel and Raju, 2014). The high levels of resistin in GCF can be explained by the main source of resistin being neutrophils, macrophages and monocytes with very little expressed in adipocytes in human (Patel et al., 2003, Rosen and Spiegelman, 2006). Indeed, this statement is corroborated by a previous study reported no association between serum or salivary resistin levels with BMI (Mamali et al., 2012). However, another study reported higher serum resistin with obesity (Piestrzeniewicz et al., 2008).

### 4.3.2 Biomarkers of tissue remodelling

It has been shown that remodelling of the ECM accompanies the expansion of adipose tissue, with enlargement of fat cells affected by MMPs and their endogenous tissue inhibitor (TIMPs) (Halberg et al., 2008). These enzymes have also been implicated in the bone and connective tissue remodelling during OTM (Nahm et al., 2004, Tiranathanagul et al., 2004) and delayed or inhibited by MMP inhibitors (Holmbeck et al., 2004). MMP8 and MMP9 are involved in different steps of collagen turnover, catalysing the initial degradation of major ECM proteins (Pilcher et al., 1999, Quasnichka et al., 2005).

In this study, due to the absence of evidence of plaque and signs of gingival inflammation or pocket formation in both obese and normal weight groups, we have assumed that cells in the periodontium can produce these MMPs; enhanced PDL remodelling activity in the obese group results in this overexpression. The levels of circulating MMP8 were increased with obesity in subjects without orthodontic treatment, due to an increase the neutrophil activation, the main source of MMP8, in obese subjects. However, the levels of MMP9 were comparable in UWMS, GCF and serum of the obese and normal weight groups (Figure 4.3), which was confirmed by the zymography results

observed by the analyses of UWMS of both obese and normal weight groups (Table 4.3, Figure 4.5). These findings agree with a previous study conducted to compare the levels of circulating MMPs and TIMPs, and their ratios in obese children and adolescents. They showed that the levels of MMP8 and MMP8/TIMP1 ratio but not MMP2 and MMP9 were increased with childhood obesity (Belo et al., 2009). Thus, obese subjects, at least in part, have a high risk for developing cardiovascular diseases, due to high circulating levels of MMP8, as several other studies reported high levels of circulating MMP8 associated with the presence and severity of cardiovascular diseases (Turu et al., 2006, Souza-Costa et al., 2007), as well as implicating them in inflammatory disorders such as metabolic syndrome (Koh et al., 2005).

In the cohort with orthodontic treatment, the levels of MMP9 were increased in both GCF and serum of the obese group could be associated with the high degradation of elastin and collagen IV, V and X (Tayebjee et al., 2004a, Tayebjee et al., 2004b) that may be increased during orthodontic treatment and enhanced by inflammatory conditions of obesity. However, the same levels of MMP8 in both obese and normal weight groups is probably due to a large number of neutrophils recruited and activated with orthodontic treatment irrespective of BMI (Figure 4.12).

Interestingly, the levels of the inhibitor TIMP1 were increased in serum of obese individuals with and without orthodontic treatment. Potentially, this is in order to balance the effects of MMPs resulting in comparable levels of MMP8/TIMP1 and MMP9/TIMP1 ratios in both cohorts. The balance between MMPs and TIMPs is a key factor that regulates matrix remodelling, and alteration of this equilibrium may contribute to many diseases (Goldberg et al., 1992).

From our data, we can assume that changes in MMP8 are mainly driven by obesity, whilst MMP9 is mainly altered during OTM. This might be a reflection of the different collagen fibre types targeted by each MMP.

### 4.3.3 Biomarker of bone remodelling

Given the central involvement of bone remodelling/metabolism in orthodontic treatment and the impact of obesity on bone metabolism, it is unsurprising that there are changes in bone metabolism in our groups. Interestingly, significant differences were found between obese and normal weight groups.

In the control cohort without orthodontic treatment, the significantly higher levels of RANKL (a key of osteoclastogensis and bone resorption) in the GCF and serum of obese subjects (Figure 4.6) can be attributed to the effects of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 that are produced by adipocytes. These findings come in line with the results of studies demonstrated that with obesity OPG/RANKL ratio produced by osteoblast was reduced, while RANK produced by osteoclast was upregulated that in turn stimulate osteoclastogensis (Halade et al., 2011, Xu et al., 2013). Also an in vitro study reported that RANKL mRNA was upregulated by the cytokines produced by adipocytes in consistence with osteoclasts differentiation results in more osteoclastogensis and bone resorption in obese subjects (Goto et al., 2011).

However, in UWMS the levels of RANKL were reduced in the obese group which possibly indicates that obesity might affect the diffusion of RANKL from serum via GCF. Also this could be linked to the wide range of cytokines produced by adipocytes, such as interleukins, which may induce or inhibit other cytokines in saliva of obese subjects.

In the orthodontic treatment cohort, although greater bone remodelling takes place, circulating RANKL was undetectable in both obese and normal weight groups (Figure 4.14); this result is surprising as more bone resorption was expected during orthodontic treatment. A possible explanation for these results may be that root resorption takes place after months of orthodontic treatment. The protective mechanism of root resorption involves production of more OPG (pseudo receptors bind with RANKL to prevent osteoclastogensis and bone resorption) by cementoblasts and other periodontal tissue to protect cementum from resorption. Therefore, the levels of RANKL in this study seems to be affected by additional factors and not wholly dependent on BMI.

Furthermore, the high levels of RANKL in GCF compared to UWMS and serum could be related to the expression of RANKL on T and B lymphocytes in human periodontal tissues, which confirmed by immunohistochemistry and confocal microscopic analyses in a previous study with periodontitis (Taubman et al., 2005).

### 4.3.4 Biomarkers of inflammation

Since the MPO enzyme is stored in neutrophil granules and released with neutrophil activation, the significantly higher levels of MPO in the UWMS, GCF and serum of obese subjects without orthodontic treatment (Figure 4.7), suggest that neutrophils are activated

via inflammatory processes linked with obesity- as confirmed by the findings of Nijhuis et al. (2009). However, in subjects with orthodontic treatment comparable levels of MPO were observed in UWMS, GCF and serum of both obese and normal weight groups, suggesting that greater numbers of neutrophils are activated as a result of increased inflammatory process associated with OTM, irrespective to BMI (Figure 4.15). Additionally, the highest level of MPO in control cohort was observed in GCF, whilst in the group with orthodontic treatment the peak was in serum. This is probably due to the inflammation associated with long time orthodontic treatment (as the sample was collected at the final alignment stage of the treatment) which may influence the circulating levels of MPO.

The other inflammatory biomarker we analysed was CRP. Notably, in our study there is an absence of any statistically significant difference of CRP levels in UWMS and GCF between the normal weight and obese subjects with and without orthodontic treatment. This could be related to the healthy gingival and periodontal status of the participants show similarly low levels of CRP in GCF and UWMS in both obese and normal weight groups. These finding are confirmed by previous studies found a correlation between CRP in saliva with the severity of periodontitis (Pederson et al., 1995) and clinical attachment loss (Aurer et al., 2005). The systemic origin and low level of CRP in GCF (when compared to serum) may go some way in explaining its low levels in UWMS in this study.

The circulating CRP in control cohort without orthodontic treatment is significantly increased in obese group compared to normal weight (Figure 4.7). These findings match the results of previous studies conducted to find out the differences between obese and non-obese participants. They found higher CRP levels in serum of obese subjects due to the inflammatory conditions linked to obesity (Ellulu et al., 2016) and increased levels of pro-inflammatory cytokines, such as IL-6 (Jaye and Waites, 1997, Du Clos, 2000). Furthermore, a significant correlation between IL-6 and CRP with BMI was outlined by Khaodhiar et al. (2004). The close association of these factors can be explained by the observation that increases in IL-6 stimulate the production of CRP by the liver (Berg and Scherer, 2005). Previous studies have also found significantly higher serum CRP in diseased groups when compared to healthy groups (Sibraa et al., 1991, Tüter et al., 2007).

In the orthodontic treatment cohort, the levels of circulating CRP were comparable in both obese and normal weight groups (Figure 4.15). This can be explained as the

orthodontic forces increase the production of pro-inflammatory cytokines in particular IL-6 in both groups, which have the potential to in turn enhance production of CRP from liver (Berg and Scherer, 2005, Ide et al., 2004, Gabay, 2006, Nibali et al., 2007). Therefore, after a long time with orthodontic treatment the levels of CRP in serum of both normal weight and obese patients can be increased independent to body weight.

### 4.3.5 Clinical implications

Statistical implication is used to test the null hypothesis at a selected level of significance. Accordingly, the levels of some biomarkers in some bio-fluids were statistically different between normal weight and obese participants in both control and orthodontic treatment cohorts. However, although these significant differences provide evidence about the biochemical changes to be taken into consideration in the long term, they give no clear indication of the magnitude or clinical importance of the difference. For instance, the significantly increased GCF flow rate in participants of our study was still within the normal range between 0.5-1 µl as reported by Lamster et al. (1985). Also our results of salivary and GCF levels of biomarkers associated with obesity and/or orthodontic treatment were incomparable to the previously published reports for the same biomarkers under pathological conditions such as periodontitis (Kinney et al., 2014).

## 4.4 Conclusion

This cross sectional study investigated the effect of obesity on inflammation, tissue and bone remodelling, and obesity-related biomarkers in UWMS, GCF and serum of normal weight and obese 18-45 years old subjects of two cohorts. The first cohort was a cohort without orthodontic treatment and the second cohort was orthodontic treatment patients having 0.019 x 0.025-inch stainless steel rectangular archwires in upper and lower fixed orthodontic appliance during the final alignment stage. This study concluded:

1- In the absence of orthodontic treatment, obesity affects markers in bio-fluids. However, orthodontic treatment produces larger effects;
2- Obesity increases the risk of inflammation and bone resorption by suppressing anti-inflammatory (adiponectin) and enhancing pro-inflammatory (MPO, CRP, RANKL) mediators. However, with good plaque and gingival control, obesity alone

is insufficient to alter the local tissue remodelling biomarkers MMP8, MMP9 and TIMP1;

3- After months of orthodontic treatment with fixed appliances, the effects of orthodontic forces are more significant than those of obesity on biomarker levels;
4- Obesity may affect the diffusion of biomarkers from serum to saliva through GCF, such as leptin and RANKL; and
5- Saliva cannot substitute GCF or serum for investigation of changes in biomarker with obesity and/or orthodontic treatment.

# Chapter 5 Clinical and biochemical effects of obesity on OTM: a prospective cohort study

## 5.1 The clinical impact of obesity on orthodontic alignment in adolescents

### 5.1.1 Introduction

OTM takes place as a direct consequence of tissue and bone remodelling following the application of external mechanical force (Meikle, 2006). It is initially represented by simple mechanical displacement of the tooth and bone-bending within the socket, which occurs due to compression of the PDL following the application of orthodontic force. However, over the longer-term tooth movement occurs as a direct consequence of connective tissue remodelling within the periodontium and alveolar bone mediated through a localised inflammatory response. This triggers the release of biochemical mediators that are essential for this process to occur and are often detectable within GCF (Kapoor et al., 2014). In particular, RANKL, MMPs and TIMPs (Grant et al., 2013).

Obesity is also known to influence systemic bone metabolism through complex mechanical, hormonal, and inflammatory interactions (López-Gómez et al., 2016) with associations between obesity and reduced bone remodelling (Ivaska et al., 2016) and increased bone mineral density (Salamat et al., 2016).

The potential implications of adolescent obesity for orthodontic treatment have been highlighted (Neeley and Gonzales, 2007) with increased BMI being a risk factor for less cooperation and longer treatment duration with fixed appliances (von Bremen et al., 2015). In addition, there is some evidence of variation in the levels of adipokines in the GCF of obese and normal weight individuals with periodontal disease, particularly up-regulation of pro-inflammatory adipokines (Zimmermann et al., 2013, Duzagac et al., 2015, Gonçalves et al., 2015, Suresh et al., 2016). However, despite known associations between raised BMI and chronic inflammatory changes within the periodontium, there have been no prospective investigations of OTM in obese patients.

#### 5.1.1.1 Aims and objectives

The main aim of this prospective cohort study was to investigate the effect of obesity on OTM in adolescent patients during routine treatment with fixed-appliances; specifically, the time taken to complete tooth alignment and variation in clinical parameters, including GCF biomarkers.

The following measurements were carried out in both normal weight and obese groups:

1- Time taken to complete tooth alignment;
2- Rates of tooth movement;
3- Variation in clinical parameters, including plaque and gingival indices, and GCF flow rate; and
4- Biochemical analyses of GCF.

The null hypothesis was that there is no difference in the time to orthodontic tooth alignment between normal weight and obese patients.

### 5.1.2 Methods

#### 5.1.2.1 Ethical approval

Ethical approval was obtained by the United Kingdom National Research Ethics Service, NRES Committee foundation (14/LO/0769, October 2014), and written informed consent was received from all parents, guardians and children. We report and present data according to the STROBE statement (Von Elm et al., 2008).

#### 5.1.2.1 Inclusion Criteria

Eligibility included the following criteria: (1) patients undergoing routine fixed-appliance treatment with or without teeth extraction; (2) 12-18 years old at treatment-start; (3) no medical contraindications or regular medication (including antibiotic-therapy in previous three-months); (4) non-smokers; (5) permanent dentition; (6) mandibular arch incisor irregularity index of 4-12 mm; and (7) normal weight (BMI-centile 2-91) and obese (BMI-centile >98) classification. Those classified as underweight (BMI-centile <2) and overweight (BMI-centile 91-98) were excluded, respectively.

### 5.1.2.2 Exclusion Criteria

Patients with a complete bite that could interfere with fixed appliance placement during the early stages of treatment; those who needed expansion with or without interdental stripping; and those who have any history of juvenile periodontal disease were excluded from the study.

### 5.1.2.3 Participants

Participants fulfilling the inclusion criteria were recruited as described in Section 2.4 number 3, between January 2015 and January 2016. Follow-up took place between January 2015 and June 2016 and covered periods from baseline records, appliance placement to completion of tooth alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch).

Following informed consent, BMI-centile was measured as described in Section 2.2.2. All measurements were taken by a single-trained operator (HFS) using the same equipment. Subjects were classified as normal weight (BMI-centile 2-91) and obese (BMI-centile >98). Those classified as underweight (BMI-centile <2) and overweight (BMI-centile 91-98) were excluded, respectively.

### 5.1.2.4 Data collection

Data were collected at four time-points as follow:

| | |
|---|---|
| **T1 ( Prior to treatment)** | • Periodontal health indices<br>• Unstimulated whole mouth saliva sample<br>• Gingival crevicular fluid sample<br>• Peripheral blood sample<br>• Dental study cast for lower arch |
| **T2 (1 hour post appliance placement)** | • Unstimulated whole mouth saliva sample<br>• Gingival crevicular fluid sample<br>• Peripheral blood sample |
| **T3 ( 1 week post appliance placement)** | • Periodontal health indices<br>• Unstimulated whole mouth saliva sample<br>• Gingival crevicular fluid sample<br>• Peripheral blood sample<br>• Dental study cast for lower arch |
| **T4 (End of the alignment)** | • Periodontal health indices<br>• Unstimulated whole mouth saliva sample<br>• Gingival crevicular fluid sample<br>• Peripheral blood sample<br>• Dental study cast for lower arch |

### 5.1.2.5 Periodontal health

Periodontal health was measured clinically using established validated gingival and plaque indices (Silness and Löe, 1964, Löe and Silness, 1963) as described in Section 2.3.

### 5.1.2.6 Impression taking

Alginate impressions of the lower arch were taken at T1, T3 and T4 in order to monitor tooth movement. The impression was then disinfected and moved to the laboratory for model casting and scanning using three-dimensional scanner (3Shape R700 laboratory scanner, Copenhagen, Denmark).

### 5.2.2.7 Alignment and rate of tooth movement

Tooth alignment was calculated from scanned dental stone casts using an irregularity-index, which measures the horizontal linear contact-point displacement of each mandibular incisor from the adjacent tooth and therefore, represents the sum of the five individual displacements (Little, 1975) (Figure 5.1).

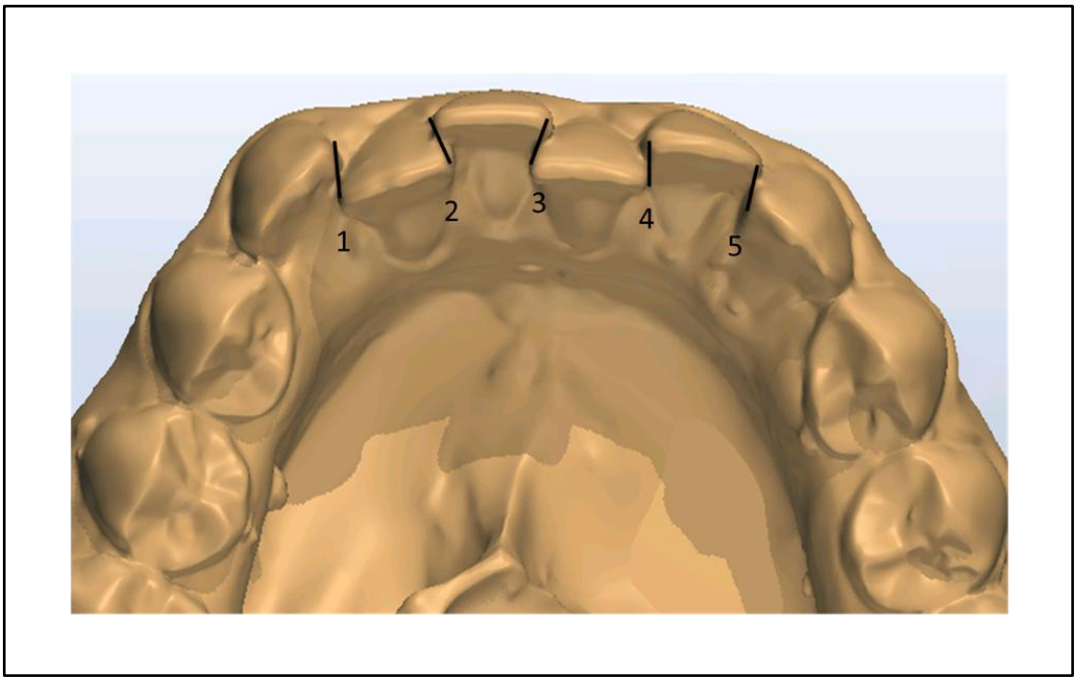



**Figure 5.1** Little's Irregularity Index to calculate teeth irregularity by measuring the liner distances (1-5) between the contact points of lower anterior teeth. Adapted from (Little, 1975).

### 5.1.2.8 Sample collection

GCF was collected as described in Section 2.4 and flow rate of GCF was measured as mentioned in Section 2.6.2.

### 5.1.2.9 Biochemical analyses

GCF was analysed by magnetic Luminex screening assay using a commercially available kit (R&D Systems, Bio-Techne, Abingdon, UK) as mentioned in Section 2.13 for detection of the selected biomarkers (in pg/ml), including the adipokines adiponectin, leptin and resistin; the inflammation biomarkers MPO and CRP; the tissue remodelling biomarkers MMP8 and MMP9, TIMP1, MMP8/TIMP1, MMP9/TIMP1; and bone remodelling biomarker RANKL.

### 5.1.2.10 Appliance placement

Fixed-appliances and bonding method were standardized between groups (3M Victory-APC 0.022-inch brackets, MBT prescription, 3M-Unitek, Monrovia, USA). After bracket bonding, a 0.014-inch nickel-titanium archwire was tied in and ligated using conventional elastomerics. The archwire was cut distal to the first molar teeth and not cinched. No bite planes, auxiliary-arches, inter-maxillary elastics, headgears or temporary anchorage devices were used during the investigation.

All appliances were placed by postgraduate orthodontic trainees under direct supervision of a consultant orthodontist. This was a pragmatic study undertaken in a clinical department. Patients were seen at approximately 6 week intervals for appliance adjustment and archwire progression took place as deemed clinically appropriate by the treating clinicians.

The primary outcome was time to achieve tooth alignment in the lower arch. Secondary outcomes included rate of tooth movement, changes in clinical parameters and GCF biomarkers during treatment. There were no changes to outcomes following study commencement.

All primary data were coded so that the outcome assessor (HFS) and statistician (SNP) were blinded to subject classification. Data-coding was broken after the end of the analysis and no blinding breaches were identified.

### 5.1.2.11 Reliability of the measurement

To examine measurement reliability and agreement, 36-pairs of models from baseline (T1) and 1 week (T3) were selected and re-measured after 2 weeks. The Concordance Correlation Coefficient (CCC) (Lawrence and Lin, 1989) and Bland-Altman method (Bland and Altman, 1986) were used to test intra-examiner reliability and agreement.

### 5.1.2.12 Study size

The sample size was based upon previous randomised prospective data on time to completion of alignment with fixed-appliances, which found a mean time-to-alignment of 200.7 days with standard deviation (SD) 73.6 days in the presence of 8.9 mm incisor irregularity (Woodhouse et al., 2015a). A total of 50 patients were required to detect with an unpaired t-test a hypothesized 30% reduction (Schulz and Grimes, 2005) in alignment

time with a common SD across groups to yield 80% power at p=0.05. Five additional patients were recruited to account for possible drop-outs.

#### 5.1.2.13 Statistical analysis

Descriptive statistics were calculated after checking for normal distribution. All biomarker data were $Log_{10}$ transformed for normalization. Initial crude differences in baseline and outcome data were calculated with independent t-tests, chi-square tests or Mann-Whitney U tests, where needed.

The effect of obesity was investigated using univariable (crude) and multivariable generalized estimation equation regression models with robust standard errors to take into account correlation between repeated measurements for each patient through the follow-up period (baseline, 1-hour, 1- week, and completion of alignment), adjusted for the confounding effect of baseline data (sex, age, and baseline-irregularity). Results are reported as unstandardized coefficients or Odds Ratios (ORs) for continuous and binary outcomes, respectively. The effect modification of obesity on the progress of tooth alignment was tested by introducing interaction terms, which were ultimately dropped from the model if not significant. Analysis of residuals was conducted to confirm the regression assumptions. As patients within the study had initial irregularity ranging from 4-12 mm, sensitivity analyses were conducted to include only those with severe (≥7 mm) or moderate (4-7 mm) baseline-irregularity. All analyses were carried out using Stata 12.0 (Statacorp College Station, Texas, USA). A 2-tailed p-value of 0.05 was considered statistically significant with a 95% confidence interval (CI) for all tests.

## 5.1.3 Results

### 5.1.3.1 Participants

The 82 patients who fulfilled the inclusion criteria were enrolled in this study, 19 patients were excluded because of changes in treatment plan, such as starting the treatment of upper arch only or add accessories for the appliance; and 8 patients were declined to participate. Therefore, this prospective cohort study included 55 patients (27 male, 28 female) with a mean age of 15.1 (SD 1.7) years and mean irregularity index of 7.6 (SD 2.4; 95% CI 6.9-8.2) mm. Mean BMI centile of the cohort was 24.7 (SD 6.2) $kg/m^2$. From the original 55 patients recruited, 7 were excluded at 1-week (T3) due to missed appointments, but all 55

were included at completion of alignment (T4). Missingness at 1-week (T3) was judged as random (Table 5.1). The reliability and agreement of repeated measurements was excellent (CCC: 0.99 with 95% CI: 0.98 to 0.99; average difference 0.06 with 95% limits of agreement: -0.68 to 0.79). Patient-flow through the study is shown in Figure 5.2.

Table 5.2 shows demographics and GCF parameters for the two cohorts at baseline (T1). The mean BMI was 19.4 (2.2) kg/m$^2$ in the normal weight group and 30.2 (3.5) kg/m$^2$ in the obese group. Apart from BMI, there were no statistically significant differences in demographics among groups at baseline; however, the obese group did have 1.2 mm more irregularity (p=0.061). In contrast, significant differences were present between normal weight and obese groups for a number of GCF biomarkers at baseline (Table 5.2; p<0.05) including increased GCF flow-rate, increased leptin, resistin, MPO, MMP8, TIMP1, RANKL, and reduced MMP9/TIMP1 levels in obese patients.

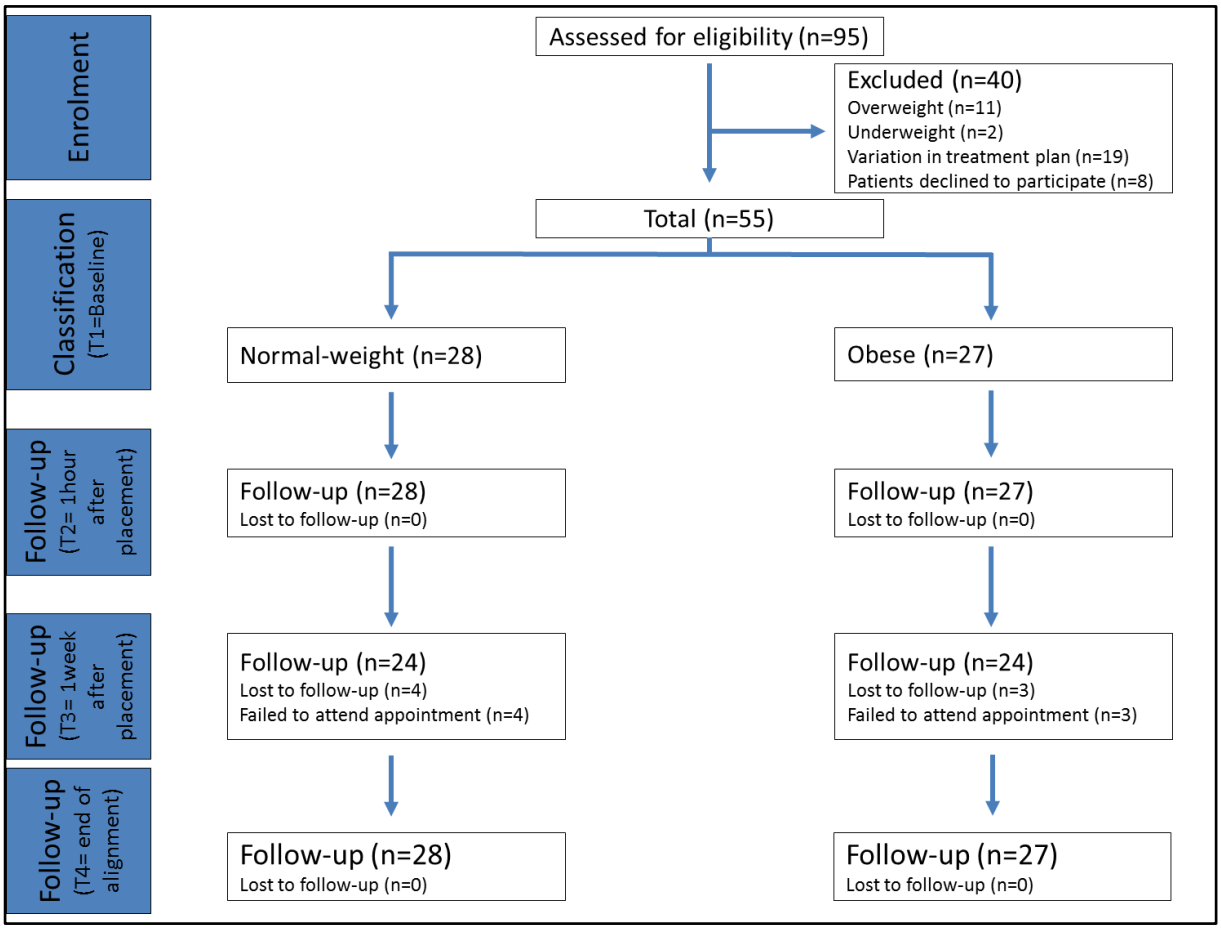



**Figure 5.2** Patient-flow through the investigation.

**Table 5.1** Demographics of the initially-recruited sample at baseline and the sample after patient drop-outs at one week.

| | **Overall** | **Normal weight** | **Obese** | **P value** |
|---|---|---|---|---|
| Recruited sample (data for baseline, completion of alignment) | | | | |
| Patients | 55 | 28 | 27 | |
| Male - n (%) | 27 (49%) | 15 (54%) | 12 (44%) | 0.498* |
| Age - mean (SD) | 15.1 (1.7) | 15.1 (1.6) | 15.1 (1.9) | 0.991# |
| Irregularity - mean (SD) | 7.6 (2.4) | 7.0 (2.3) | 8.2 (2.4) | 0.061# |
| BMI - mean (SD) | 24.7 (6.2) | 19.4 (2.2) | 30.2 (3.5) | <0.001# |
| Sample with drop-outs (data for one week) | | | | |
| Patients | 48 | 24 | 24 | |
| Male - n (%) | 24 (50%) | 13 (54%) | 11 (46%) | 0.564* |
| Age - mean (SD) | 15.3 (1.7) | 15.2 (1.6) | 15.3 (1.8) | 0.737# |
| Irregularity - mean (SD) | 7.7 (2.4) | 7.2 (2.3) | 8.2 (2.5) | 0.156# |
| BMI - mean (SD) | 24.7 (6.2) | 19.3 (2.2) | 30.2 (3.6) | <0.001# |

SD, standard deviation; BMI, body mass index; * from chi-square test; # from independent t-test.

**Table 5.2** Demographics and GCF parameters of patients at baseline.

| | | **Overall** | **Normal weight** | **Obese** | **P value** |
|---|---|---|---|---|---|
| Demographics | | | | | |
| | Patients (n) | 55 | 28 | 27 | |
| | Male / female (n) | 27/28 | 15/13 | 12/15 | 0.498* |
| | Age - mean (SD) | 15.1 (1.7) | 15.1 (1.6) | 15.1 (1.9) | 0.991# |
| | Caucasian – n (%) | 29 (61) | 17 (61) | 12 (44) | 0.516* |
| | Asian – n (%) | 4 (7) | 2 (7) | 2 (7) | |
| | African – n (%) | 5 (9) | 1 (4) | 4 (15) | |
| | Mixed – n (%) | 8 (15) | 3 (11) | 5 (19) | |
| | Others – n (%) | 9 (16) | 5 (18) | 4 (15) | |
| | BMI - mean (SD) | 24.7 (6.2) | 19.4 (2.2) | 30.2 (3.5) | **<0.001**# |
| | Plaque index (SD) | 0.56 (0.32) | 0.57 (0.32) | 0.54 (0.31) | 0.745# |
| | Gingival index (SD) | 0.74 (0.39) | 0.74 (0.40) | 0.73 (0.38) | 0.934# |
| | Irregularity - mean (SD) | 7.6 (2.4) | 7.0 (2.3) | 8.2 (2.4) | 0.061# |
| | Severe irregularity¥ – n (%) | 31 (56%) | 13 (46%) | 18 (67%) | 0.130* |
| | Tooth extraction – n (%) | 8 (15%) | 4 (14%) | 4 (15%) | 0.956* |
| GCF parameters ($Log_{10}$ transformed) | | | | | |
| | GCF flow-rate † (µl/min) | -0.29 (0.14) | -0.33 (0.12) | -0.25 (0.12) | **0.011**£ |
| | Adiponectin (pg/ml) | 6.60 (0.34) | 6.55 (0.42) | 6.66 (0.23) | 0.237# |
| | Leptin‡ (pg/ml) | 13.91 (22.64) | 6.40 (14.65) | 19.15 (24.45) | **0.031**£ |
| | Resistin (pg/ml) | 5.61 (0.56) | 5.30 (0.55) | 5.92 (0.36) | **<0.001**# |
| | MPO (pg/ml) | 5.05 (0.85) | 4.44 (0.64) | 5.69 (0.52) | **<0.001**# |
| | CRP† (pg/ml) | 2.50 (0.51) | 2.47 (0.27) | 2.65 (0.87) | 0.827# |
| | MMP8 (pg/m) | 6.32 (0.64) | 6.01 (0.48) | 6.64 (0.64) | **<0.001**# |
| | MMP9† (pg/ml) | 6.23 (0.30) | 6.18 (0.59) | 6.27 (0.15) | 0.245# |
| | TIMP1 (pg/ml) | 5.00 (0.48) | 4.72 (0.38) | 5.28 (0.40) | **<0.001**# |
| | MMP8/TIMP1 (pg/ml) | 0.96 (0.64) | 0.93 (0.62) | 0.99 (0.68) | 0.699# |
| | MMP9/TIMP1 (pg/ml) | 0.54 (0.54) | 0.75 (0.54) | 0.32 (0.47) | **0.002**# |
| | RANKL† (pg/ml) | 3.54 (0.33) | 3.39 (0.25) | 3.65 (0.27) | **<0.001**£ |

SD, standard deviation; BMI, body mass index; GCF, gingival crevicular fluid; MPO, myeloperoxidase; CRP, C reactive protein; MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; RANKL, receptor activator of nuclear factor kappa-B ligand.* from chi-square test. # from independent t-test. £ from Mann-Whitney test. ¥ according to Little (1975). †Log10 transformation improved the skewness of the data, but the Shapiro-Wilk test indicated that transformed data were still not normally distributed. Therefore, the median (interquartile range) is presented instead of mean (SD) and the Mann-Whitney test is used on the transformed data instead of the unpaired t-test. ‡ Square root transformation used instead of log10, as several null values were included. Therefore, the median (interquartile range) is presented instead of mean (SD) and the Mann-Whitney test is used on the transformed data instead of the unpaired t-test.

### 5.1.3.2 Primary outcome

The results of both crude and adjusted regression analyses indicated only a small difference in time to achieve alignment between obese and normal weight patients. Overall, obese patients needed a mean 23.0 days less than normal weight to reach final alignment (Figure 5.3 A), but this was not statistically significant (Table 5.3, $p>0.05$).

### 5.1.3.3 Secondary outcomes

A number of significant differences were observed between obese and normal-weight patients in the clinical response to orthodontic force. The rate of mechanical tooth displacement within week-1 was statistically significantly increased in the obese group ($p<0.001$); whilst overall rate of alignment from baseline to completion-of-alignment was also increased ($p=0.05$) (Table 5.4). However, tooth alignment rate from week-1 to completion-of-alignment was not significantly different between groups (Table 5.4; $p=0.119$). After taking into account all confounders in the adjusted analysis, obese patients were associated with a significantly increased rate of tooth movement throughout the whole study duration compared to normal weight patients (0.017 mm/day; 95% CI: 0.008, 0.025 mm/day). Additionally, a significant association was found between rate of tooth movement and initial tooth irregularity (0.007 mm/day increase per mm irregularity) (Table 5.3, Figure 5.3 B). Sensitivity analyses for patients with either severe or moderate irregularity were consistent in direction with the main analyses (Table 5.5 and 5.6 respectively) with an expected loss of power due to the division of the study sample, and a higher difference in alignment rate between obese and normal weight patients in the severe irregularity group.

**Table 5.3** Regression analyses on primary outcome, time to completion of tooth alignment in days, and secondary outcome, rate of orthodontic tooth movement in mm/day.

| | | | **Crude model** | | | **Adjusted model** | | |
|---|---|---|---|---|---|---|---|---|
| **Primary outcome (time to completion of tooth alignment)** | **Factor** | | **b** | **95% CI** | **P value** | **b** | **95% CI** | **P value** |
| | BMI group | Obese | -14.3 | -54.3, 25.7 | 0.483 | -23.1 | -66.1, 20.1 | 0.295 |
| | | Normal weight | Ref | | | | | |
| | Age | Per year | NT | | | -3.2 | -15.6, 9.1 | 0.608 |
| | Gender | Male | NT | | | -6.6 | -48.4, 35.2 | 0.756 |
| | | Female | NT | | | | | |
| | Ethnicity | | NT | | | -0.2 | -13.4, 13.0 | 0.974 |
| | Extraction | Yes | NT | | | -15.3 | -76.0, 45.5 | 0.623 |
| | | No | NT | | | | | |
| | Baseline irregularity | Per mm | NT | | | 6.9 | -2.50, 16.3 | 0.151 |
| | | | | | | | | |
| **Secondary outcome (rate of orthodontic tooth movement: baseline-to completion of alignment)** | **Factor** | | **b** | **95% CI** | **P value** | **b** | **95% CI** | **P value** |
| | BMI group | Obese | 0.023 | 0.011, 0.035 | <0.001 | 0.017 | 0.008, 0.025 | <0.001 |
| | | Normal weight | Ref | | | Ref | | |
| | Time* | 1 week | Ref | | | Ref | | |
| | | Complete alignment | -0.025 | -0.04, -0.015 | <0.001 | -0.021 | -0.032, 0.010 | <0.001 |
| | Age | Per year | NT | | | 0.001 | -0.002, 0.003 | 0.609 |
| | Gender | Male | NT | | | 0.001 | -0.008, 0.011 | 0.768 |
| | | Female | NT | | | Ref | | |
| | Ethnicity | | NT | | | -0.001 | -0.003, 0.002 | 0.643 |
| | Irregularity at each phase start | Per mm | NT | | | 0.007 | 0.005, 0.009 | <0.001 |
| | Extraction | Yes | NT | | | -0.004 | -0.014, 0.006 | 0.415 |
| | | No | NT | | | Ref | | |

b, unstandardized regression coefficient; CI confidence interval; BMI, body mass index; Ref, reference; NT, not tested; *Interaction of obesity with time found to be non-significant (p=0.112) and was dropped from the model.

**Table 5.4** Rate of tooth movement (mm/day) during the study period.

| Outcome | Overall (n=55) | Normal weight (n=28) | Obese (n=27) | P value |
|---|---|---|---|---|
| Time to completion of alignment (d) – mean (SD) | 158.7 (75.3) | 165.8 (72.5) | 151.4 (78.7) | 0.486 |
| Tooth alignment rate: baseline to completion of alignment (mm/d) – mean (SD) | 0.057 (0.029) | 0.050 (0.025) | 0.065 (0.031) | 0.050* |
| Tooth alignment rate: baseline to week one (mm/d) – mean (SD) † | 0.081 (0.031) | 0.065 (0.025) | 0.097 (0.028) | <0.001* |
| Tooth alignment rate: week one to completion of alignment (mm/d) – mean (SD) † | 0.056 (0.031) | 0.049 (0.027) | 0.063 (0.033) | 0.119* |

d, days; SD, standard deviation; CI, confidence interval. * from independent t-test.
†Due to 7 patient drop-outs at one week, 48/55 patients (24 obese and 24 control patients) are included in these two measurements. The measurement of time-to-alignment and alignment rate: baseline to completion of alignment pertains to the whole sample of 55 patients.

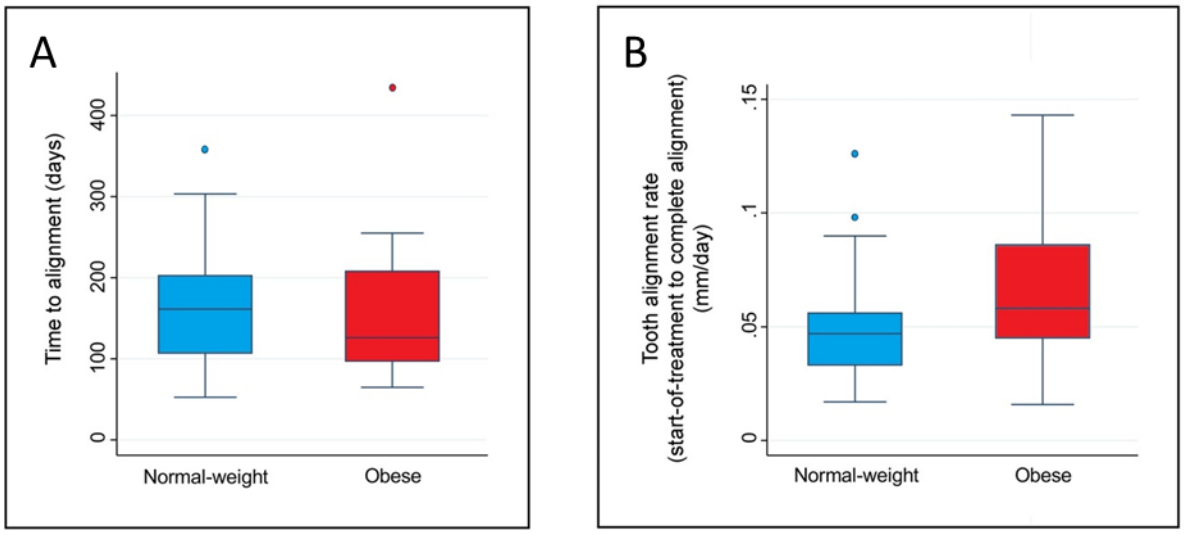



**Figure 5.3** Box plots of measured values in normal weight and obese patients for (A) primary (time to completion of alignment in days) and (B) secondary (tooth alignment rate from start of treatment to completion of alignment in mm/day) outcomes. Plotted boxes with horizontal lines indicate interquartile ranges with medians. Vertical whiskers and points indicate upper and lower adjacent values and outliers.

**Table 5.5** Sensitivity analysis: regression analysis on the primary (time to completion of alignment in days) and secondary outcome (rate of tooth movement in mm/day) for the category of patients with severe initial crowding (≥7 mm; n=31).

| | | | | Crude model | | | Adjusted model | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sensitivity analysis: initial crowding ≥7 mm (n=31)** | | **Factor** | | **b** | **95% CI** | **P value** | **b** | **95% CI** | **P value** |
| | **Primary outcome: time to alignment** | BMI group | Obese | -16.0 | -67.9, 35.9 | 0.545 | -16.1 | -77.6, 45.3 | 0.607 |
| | | | Normal weight | Ref | | | Ref | | |
| | | Age | Per year | NT | | | -3.8 | -18.9, 11.2 | 0.617 |
| | | Gender | Male | | | | 11.6 | -46.4, 69.6 | 0.695 |
| | | | Female | NT | | | Ref | | |
| | | Ethnicity | | NT | | | -5.9 | -24.0, 12.3 | 0.527 |
| | | Irregularity at baseline | Per mm | NT | | | 17.3 | -2.4, 37.0 | 0.085 |
| | | Extraction | Yes | | | | -11.2 | -74.8, 52.5 | 0.731 |
| | | | No | NT | | | Ref | | |
| | | | | | | | | | |
| | | **Factor** | | **b** | **95% CI** | **P value** | **b** | **95% CI** | **P value** |
| | **Secondary outcome: alignment rate** | BMI group | Obese | 0.022 | 0.009, 0.035 | 0.001 | 0.021 | 0.008, 0.033 | 0.002 |
| | | | Normal weight | Ref | | | Ref | | |
| | | Time point * | 1 week | Ref | | | Ref | | |
| | | | Complete alignment | -0.033 | -0.048, -0.018 | <0.001 | -0.029 | -0.046, 0.0129 | <0.001 |
| | | Age | Per year | NT | | | 0.001 | -0.003, 0.005 | 0.614 |
| | | Gender | Male | | | | 0.003 | -0.009, 0.015 | 0.625 |
| | | | Female | Ref | | | Ref | | |
| | | Ethnicity | | NT | | | -0.000 | -0.005, 0.004 | 0.828 |
| | | Irregularity at each phase start | Per mm | NT | | | 0.005 | 0.000, 0.010 | 0.037 |
| | | Extraction | Yes | | | | -0.001 | -0.014, 0.012 | 0.910 |
| | | | No | Ref | | | Ref | | |

b, unstandardized regression coefficient; CI, confidence interval; Ref, reference.
*Interaction of obesity with time found to be non-significant (p=0.171) and was dropped from the model.

**Table 5.6** Sensitivity analysis: regression analysis on the primary (time to completion of alignment in days) and secondary outcome (rate of tooth movement in mm/day) for the category of patients with moderate initial crowding (<7mm; n=24).

| | | | | Crude model | | | Adjusted model | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sensitivity analysis: initial crowding <7 mm (n=24)** | | **Factor** | | **b** | **95% CI** | **P value** | **b** | **95% CI** | **P value** |
| | **Primary outcome: time to alignment** | BMI group | Obese | -18.04 | -85.68, 49.59 | 0.601 | 2.7 | -78.2, 83.6 | 0.948 |
| | | | Normal weight | Ref | | | Ref | | |
| | | Age | Per year | NT | | | 8.1 | -17.8, 34.0 | 0.540 |
| | | Gender | Male | | | | -40.2 | -117.1, 36.7 | 0.305 |
| | | | Female | NT | | | Ref | | |
| | | Ethnicity | | NT | | | 8.2 | -14.3, 30.8 | 0.474 |
| | | Irregularity at baseline | Per mm | NT | | | 7.2 | -34.1, 48.4 | 0.734 |
| | | Extraction | Yes | | | | -32.7 | -227.6, 162.2 | 0.742 |
| | | | No | NT | | | Ref | | |
| | | | | | | | | | |
| | | **Factor** | | **b** | **95% CI** | **P value** | **b** | **95% CI** | **P value** |
| | **Secondary outcome: alignment rate** | BMI group | Obese | 0.013 | -0.002, 0.028 | 0.100 | 0.013 | 0.001, 0.025 | 0.039 |
| | | | Normal weight | Ref | | | Ref | | |
| | | Time point * | 1 week | Ref | | | Ref | | |
| | | | Complete alignment | -0.015 | -0.028, -0.001 | 0.030 | -0.012 | -0.026, 0.003 | 0.117 |
| | | Age | Per year | NT | | | -0.001 | -0.005, 0.002 | 0.484 |
| | | Gender | Male | | | | 0.003 | -0.009, 0.014 | 0.657 |
| | | | Female | Ref | | | Ref | | |
| | | Ethnicity | | NT | | | -0.001 | -0.005, 0.002 | 0.491 |
| | | Irregularity at each phase start | Per mm | NT | | | 0.007 | -0.001, 0.015 | 0.104 |
| | | Extraction | Yes | | | | -0.005 | -0.020, 0.009 | 0.477 |
| | | | No | Ref | | | Ref | | |

b, unstandardized regression coefficient; CI, confidence interval; Ref, reference.
*Interaction of obesity with time found to be non-significant (p=0.171) and was dropped from the model.

Plaque and gingival indices deteriorated significantly (Table 5.7), but there were no differences between groups for any of these parameters. GCF flow-rate increased during orthodontic treatment for both groups, but significantly more in obese patients.

**Table 5.7** Results of regression models assessing the effect of obesity and time-variation during orthodontic treatment on the levels of clinical indices and GCF biomarkers. Explorative interactions between obesity and time were tested in each case, but were dropped from the model if they were not statistically significant.

| Biomarker transformation | Factor | | b | 95% CI | P value |
|---|---|---|---|---|---|
| Plaque index | Obese | | 0.03 | -0.05,0.12 | 0.460 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | - | | |
| | | T3 | 0.16 | 0.07,0.26 | 0.001 |
| | | T4 | 0.34 | 0.27,0.41 | <0.001 |
| | Interaction | | | | 0.353 |
| Gingival index | Obese | | 0.04 | -0.05,0.12 | 0.391 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | - | | |
| | | T3 | 0.14 | 0.06,0.21 | 0.001 |
| | | T4 | 0.30 | 0.24,0.36 | <0.001 |
| | Interaction | | | | 0.206 |
| GCF flow-rate | Obese | | 0.08 | 0.04,0.13 | <0.001 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.10 | 0.05,0.15 | <0.001 |
| | | T3 | 0.10 | 0.06,0.14 | <0.001 |
| | | T4 | 0.09 | 0.04,0.13 | <0.001 |
| | Interaction | | | | 0.282 |
| Adiponectin | Obese | | 0.08 | -0.12,0.28 | 0.417 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.15 | 0.04,0.26 | 0.010 |
| | | T3 | 0.23 | 0.10,0.36 | <0.001 |
| | | T4 | 0.15 | 0.01,0.28 | 0.031 |
| | Interaction | | -0.07 | -0.13,-0.01 | 0.027 |
| Leptin | Obese | | 6.41 | 1.71,11.10 | 0.007 |
| Square-root transformed | Time | T1 | Ref | | |
| | | T2 | -2.44 | -5.18,0.29 | 0.080 |
| | | T3 | -1.21 | -1.85,4.27 | 0.439 |
| | | T4 | -4.44 | -8.50,-0.38 | 0.032 |
| | Interaction | | | | 0.556 |

Continue Table 5.7

| **Biomarker transformation** | **Factor** | | **b** | **95% CI** | **P value** |
|---|---|---|---|---|---|
| Resistin | Obese | | 0.60 | 0.44,0.76 | <0.001 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.13 | -0.04,0.30 | 0.124 |
| | | T3 | 0.03 | -0.16,0.21 | 0.757 |
| | | T4 | -0.05 | -0.18,0.09 | 0.519 |
| | Interaction | | | | 0.975 |
| MPO | Obese | | 1.36 | 1.11,1.62 | <0.001 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.36 | 0.13,0.59 | 0.003 |
| | | T3 | 0.56 | 0.39,0.74 | <0.001 |
| | | T4 | 0.69 | 0.52,0.87 | <0.001 |
| | Interaction | | | | 0.994 |
| CRP | Obese | | -0.02 | -0.24,0.20 | 0.860 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.10 | -0.04,0.23 | 0.149 |
| | | T3 | 0.27 | 0.09,0.46 | 0.004 |
| | | T4 | 0.26 | 0.07,0.44 | 0.006 |
| | Interaction | | | | 0.192 |
| MMP8 | Obese | | 0.62 | 0.32,0.93 | <0.001 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.16 | 0.05,0.28 | 0.007 |
| | | T3 | 0.12 | 0.01,0.23 | 0.037 |
| | | T4 | 0.05 | -0.07,0.17 | 0.446 |
| | Interaction | | | | 0.995 |
| MMP9 | Obese | | 0.06 | -0.02,0.15 | 0.149 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.38 | 0.28,0.49 | <0.001 |
| | | T3 | 0.35 | 0.25,0.44 | <0.001 |
| | | T4 | 0.08 | -0.03,0.19 | 0.181 |
| | Interaction | | | | 0.388 |
| TIMP1 | Obese | | 0.61 | 0.38,0.84 | <0.001 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | -0.04 | -0.20,0.13 | 0.668 |
| | | T3 | 0.25 | 0.07,0.42 | 0.005 |
| | | T4 | 0.42 | 0.23,0.60 | <0.001 |
| | Interaction | | -0.08 | -0.15,-0.00 | 0.040 |

Continue Table 5.7

| **Biomarker transformation** | **Factor** | | **b** | **95% CI** | **P value** |
|---|---|---|---|---|---|
| MMP8/TIMP1 | Obese | | 0.20 | -0.14,0.54 | 0.254 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.24 | 0.03,0.45 | 0.027 |
| | | T3 | -0.05 | -0.22,0.13 | 0.600 |
| | | T4 | -0.25 | -0.42,-0.08 | 0.003 |
| | Interaction | | | | 0.166 |
| MMP9/TIMP1 | Obese | | -0.34 | -0.57,-0.11 | 0.004 |
| $Log_{10}$ transformed | | | | | |
| | Time | T1 | Ref | | |
| | | T2 | 0.46 | 0.33,0.59 | <0.001 |
| | | T3 | 0.18 | 0.02,0.34 | 0.024 |
| | | T4 | -0.23 | -0.35,-0.10 | <0.001 |
| | Interaction | | | | 0.239 |
| RANKL | Obese | | 0.38 | 0.28,0.48 | <0.001 |
| $Log_{10}$ transformed | Time | T1 | Ref | | |
| | | T2 | 0.01 | -0.06,0.09 | 0.733 |
| | | T3 | 0.03 | -0.06,0.12 | 0.532 |
| | | T4 | 0.37 | 0.27,0.47 | <0.001 |
| | Interaction | | | | 0.089 |

T1, baseline; T2, 1 hour; T3, 1 week; T4, completion of alignment; b, unstandardized regression coefficient; CI, confidence interval; Ref, reference; GCF, gingival crevicular fluid; MPO, myeloperoxidase; CRP, C reactive protein; MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; RANKL, receptor activator of nuclear factor kappa-B ligand.

In order to further understand the biochemical basis of observed differences in rates of tooth movement, explorative regression analyses were undertaken (Table 5.8). GCF flow rate and levels of leptin, resistin, MPO, MMP8, TIMP1, MMP9/TIMP1 and RANKL were significantly different between obese and normal weight patients at baseline (Table 5.2) and during subsequent assays (Table 5.7).

When the grounds for a possible inter-relation between these biomarkers and rate of tooth movement were investigated, it was found that GCF levels of leptin, resistin, MPO and RANKL were significantly associated with the amount of tooth movement for each patient (Table 5.9). Therefore, from an epidemiological basis, these would be the best candidates to explain the clinical performance difference between obese and normal weight patients during fixed-appliance orthodontic alignment.

**Table 5.8** Summary of exploratory analyses on the secondary outcome: GCF biomarker levels.

| | | **Different in obese and normal weight at baseline (Table 5.2)** | **Different in obese and normal weight during treatment ( Table 5.7 )** | **Associated with alignment rate (Table 5.9 )** |
|---|---|---|---|---|
| Clinical indices | | | | |
| | Plaque index | No | No | - |
| | Gingival index | No | No | - |
| | GCF flow rate | Yes | Yes | No |
| GCF Biomarkers | | | | |
| | Adiponectin | No | No | - |
| | Leptin | Yes | Yes | Yes |
| | Resistin | Yes | Yes | Yes |
| | MPO | Yes | Yes | Yes |
| | CRP | No | No | - |
| | MMP8 | Yes | Yes | No |
| | MMP9 | No | No | - |
| | TIMP1 | Yes | Yes | No |
| | MMP8/TIMP1 | No | No | - |
| | MMP9/TIMP1 | Yes | Yes | No |
| | RANKL | Yes | Yes | Yes |

MPO, myeloperoxidase; CRP, C reactive protein; MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; RANKL, receptor activator of nuclear factor kappa-B ligand; b, unstandardised regression coefficient.

**Table 5.9** Results of regression models assessing the association of time-variation during orthodontic treatment, baseline irregularity, and biomarker levels with tooth alignment rate in mm/day.

| **Biomarker transformation** | **Factor** | **b** | **95% CI** | **P value** |
|---|---|---|---|---|
| Leptin | Time | -0.017 | -0.029,-0.005 | 0.005 |
| Square-root transformed | Baseline irregularity | 0.007 | 0.005,0.010 | <0.001 |
| | Leptin | 0.001 | 0.000,0.001 | 0.006 |
| Resistin | Time | -0.020 | -0.030,-0.010 | <0.001 |
| $Log_{10}$ transformed | Baseline irregularity | 0.007 | 0.005,0.010 | <0.001 |
| | Resistin | 0.013 | 0.006,0.020 | 0.001 |
| MPO | Time | -0.022 | -0.032,-0.012 | <0.001 |
| $Log_{10}$ transformed | Baseline irregularity | 0.007 | 0.005,0.009 | <0.001 |
| | MPO | 0.010 | 0.004,0.014 | <0.001 |
| MMP8 | Time | -0.021 | -0.031,-0.010 | <0.001 |
| $Log_{10}$ transformed | Baseline irregularity | 0.007 | 0.005,0.010 | <0.001 |
| | MMP8 | 0.003 | -0.004,0.010 | 0.415 |
| TIMP1 | Time | -0.022 | -0.032,-0.011 | <0.001 |
| $Log_{10}$ transformed | Baseline irregularity | 0.007 | 0.005,0.010 | <0.001 |
| | TIMP1 | 0.004 | -0.004,0.012 | 0.335 |
| MMP9/TIMP1 | Time | -0.022 | -0.033,-0.010 | <0.001 |
| $Log_{10}$ transformed | Baseline irregularity | 0.007 | 0.005,0.009 | <0.001 |
| | MMP9/TIMP1 | -0.003 | -0.012,0.007 | 0.592 |
| RANKL | Time | -0.025 | -0.035,-0.014 | <0.001 |
| $Log_{10}$ transformed | Baseline irregularity | 0.007 | 0.005,0.010 | <0.001 |
| | RANKL | 0.011 | 0.000,0.022 | 0.040 |

MPO, myeloperoxidase; MMP8, matrix metalloproteinase-8; TIMP1, tissue inhibitor of metalloproteinase 1; RANKL, receptor activator of nuclear factor kappa-B ligand.

## 5.1.4 Discussion

This prospective study followed a cohort of obese and normal-weight adolescent patients during the alignment phase of fixed-appliance orthodontic treatment. Obese patients needed less time to achieve alignment but this was not significant. However, obese patients demonstrated significantly increased rates of tooth movement during the whole observation period; this apparent discrepancy might be explained by a number of factors. Firstly, the obese group had a significantly increased initial mechanical displacement of the teeth during the first week following the application of orthodontic force (T1-T3), then decreased until the two groups showed comparable movement between the first week and the end of alignment (T3-T4). At the end of alignment, the obese group displayed a significantly higher rate of overall tooth movement (T1-T4) with comparable duration of teeth alignment. Also there may have been possible between-group variation in attendance during routine appointments. Evidence exists from a similar experimental model that initial alignment can increase by 0.01 mm per day whilst overall alignment increases by 0.004 mm per day every millimetre of initial irregularity (Woodhouse et al., 2015a). It is important to remember that statistical and clinical significance are not the same. A randomized controlled trial conducted to investigate the effect of bracket–ligature combination on the amount of orthodontic space closure over 3 months demonstrated a non-significant difference in the amount of space closure (1 mm per 28 days) between three different bracket/archwire combinations (Wong et al., 2013). Thus, the results of our study with a statistical significant difference of 0.017 mm per day (0.5 mm per month) would be clinically insignificant.

Significant differences were also found in the GCF biochemical profile between obese and normal weight patients and to our knowledge, this represents the first prospective data to suggest that obese patients may respond differently to those with normal weight during routine orthodontic treatment.

Appliance variation has little or no effect on rate of OTM (Woodhouse et al., 2015a, Scott et al., 2008a). Interestingly, we found that obesity does influence tooth movement, as obese patients had increased rates when compared to normal weight. Statistical modelling of alignment rate and its change through time (Table 5.3) demonstrated that obesity and initial irregularity at each phase explained part of the variation seen in alignment. Given the absence of a significant interaction between obesity and time, the difference in alignment

rate between obese and normal weight patients was consistently present through the alignment process and independent of confounders.

Importantly, the groups in this investigation were not different in terms of baseline demographics, including plaque/gingival indices and irregularity, with BMI representing the only significant difference. However, a number of differences existed in baseline GCF parameters between groups, including GCF flow-rate and the levels of several biomarkers. The pro-inflammatory adipokines leptin and resistin were both elevated in the GCF of obese patients (Suresh et al., 2016), suggestive of a baseline pro-inflammatory state within the periodontium of these individuals. It is also consistent with the significantly increased levels of MPO, which represents an established marker for inflammation in the GCF (Marcaccini et al., 2010, Navarro-Palacios et al., 2014). Interestingly, the levels of several biochemical mediators of tissue and bone remodelling were also increased at baseline in the obese group, including MMP8, TIMP1 and RANKL, providing evidence of an altered inflammatory biochemical profile in the GCF of obese patients.

Amongst the GCF biomarkers assayed, leptin, resistin, MPO, and RANKL most predictably accounted for the observed differences in rates of tooth movement. The levels of these biomarkers differed significantly between obese and normal weight patients both before and during treatment; whilst at the same time, being significantly associated with the amount of tooth movement observed.

The higher levels of leptin and resistin in GCF of obese patients may enhance the pro-inflammatory condition of those patients during orthodontic treatment. However, these biomarkers did not remarkably change at different time points of orthodontic alignment in both normal weight and obese patients, indicating that orthodontic force did not influence the levels of these adipokines in GCF. This result disagrees with a previous study, which reported reduced levels of GCF leptin during orthodontic treatment (Dilsiz et al., 2010). This inconsistency could be related to differences in BMI, age and assay used for biochemical analyses. For instance, Dilsiz and co-workers used ELISA assay to investigate leptin in GCF of normal weight teenagers. Resistin, like leptin, is up-regulated in inflamed gingival tissue as compared to healthy (Suresh et al., 2016), but the relationship between GCF resistin and OTM has not previously been investigated.

The levels of MPO increased significantly after fixed orthodontic appliance placement and continued until the end of alignment, with statistically significantly higher levels in obese patients at all-time points (T1-T4). These results reflect the greater

neutrophil activation in the periodontal tissues of obese patients, for whom obesity-related inflammation was exacerbated by orthodontic treatment. The increased levels of MPO with orthodontic treatment confirmed by previous studies reported that MPO levels were increased 2 hours after appliance placement (Marcaccini et al., 2010) with no differences between severe and moderate crowding (Navarro-Palacios et al., 2014). The high levels of MPO associated with obesity were also confirmed by recent studies reporting a significant increase in MPO levels in obese children compared to control groups (Marcovecchio et al., 2016, Varma et al., 2016).

The higher levels of RANKL in obese patients before and throughout the orthodontic alignment (T1-T4) could be related to higher pro-inflammatory cytokines produced by adipocytes such as TNF-α, IL-1β and IL-6, which stimulate RANKL/RANK pathway and result in more osteoclastogensis and bone resorption with obesity (Khosla, 2001, Pfeilschifter et al., 2002).

The other biomarker, MMP8, is mainly produced by neutrophils, demonstrated significantly higher levels in GCF of obese patients throughout the whole alignment stage. This indicates that neutrophils are highly activated by obesity, leading to higher levels of MMP8 in obese patients. Similarly, the higher levels of the inhibitor (TIMP1) in obese patients before and throughout the alignment period could be considered as an attempt to counterpart the increase in the levels of MMP8 and balance the ECM destruction by MMPs during remodelling.

The results of this study did not show any significant association between plaque and gingival indices and the levels of biomarkers at all-time points. This indicates that the variation in the levels of biomarkers in GCF resulted from the response of periodontal tissues to the applied orthodontic force rather than bacterial plaque or gingivitis.

The strengths of the present study include its prospective design (Papageorgiou et al., 2015b), baseline comparability between experimental groups, absence of drop-outs at study completion and use of measurement-blinding. Moreover, obesity was defined according to widely accepted and reliable measures, which makes misclassification unlikely. Collectively, this means that the respective risk for selection, attrition and detection bias is low. The study sample was based on a conservative a priori power calculation, and planned dropouts did not occur. However, some potential limitations include the fact that height and weight measurements were only taken at baseline and adiposity is not necessarily a static measure. Indeed, in an adolescent population underlying

growth might have influenced BMI during the course of the investigation, although with a mean observation of 158 days and mean patient age of 15.1 years, this effect may have even been negligible. In addition, only BMI was used to classify adiposity, which can limit the identification of overweight and could have been reduced by adding estimates of adiposity (body adiposity index) and fat distribution (waist-to-height ratio) (del Mar Bibiloni et al., 2013). Moreover, in order to widen the inclusion criteria of participants, the need for teeth extraction was not considered as an exclusive criterion which may have an impact on the rate of tooth movement. Also in a cohort undergoing routine orthodontic treatment with fixed appliances it is not practical to see each patient at exactly the same time-point for archwire adjustments or identify the absolute first time-point that alignment is complete for every single patient. For these reasons, the increased rates of tooth movement that were identified in the obese group may not have resulted in a clinically significant reduction in time to final alignment. In addition, it is worth mentioning that there is another statistical method for analysing the medical research data collected serially on subjects, which takes the form of a two stage method that uses summary measures (Matthews et al., 1990).

In addition, whilst the observed differences in rates of tooth movement are tangible effects with obvious potential clinical relevance, the underlying biological mechanisms are likely to be complex. The measured differences in GCF biomarkers may be associated with the inter-relationship between obesity and tooth movement, but this study provides no conclusive evidence. Further investigation will be required to elucidate the precise role of each biomarker in mediating OTM. However, this investigation provides evidence that informs clinical practice both in orthodontics and wider healthcare. The results are applicable to obese and normal weight patients, although it should be remembered that adipose tissue can behave differently according to age group in other body systems (Palmer and Kirkland, 2016). A pro-inflammatory obese state can influence OTM, with significant associations between levels of specific biomarkers within the GCF of obese patients. These results highlight potential implications for orthodontic treatment in obese subjects and one area for future research would be a comparison of post-orthodontic stability in obese and normal weight patients.

### 5.1.5 Conclusion

This prospective clinical study investigated tooth alignment in obese and normal weight patients undergoing fixed-appliance orthodontic treatment. Obese and normal weight patients needed the same time to achieve tooth alignment. After adjusting for confounders, the rate of OTM was significantly higher in obese patients compared to normal weight. Additionally, explorative analyses indicated GCF-levels of leptin, resistin, MPO and RANKL were significantly different between obese and normal weight patients and associated with observed rates of OTM. This is the first prospective data to show a different orthodontic treatment response in obese patients. These differences have potential clinical implications for treatment over both the short and long-term.

# 5.2 Time-related changes in biomarker levels during orthodontic alignment in normal weight and obese patients

## 5.2.1 Introduction

Biological reaction in the teeth and supporting tissue during OTM is a subject of interest (Meikle, 2006, Davidovitch and Krishnan, 2009). When orthodontic mechanical stimuli transfer to the teeth, blood vessels dilate and inflammatory cells are recruited to the periodontal space and produce mediators as biological response. The local micro-environmental status of the supporting tissue during orthodontic treatment can therefore be reflected by these mediators (Kook et al., 2011, Zainal Ariffin et al., 2011, Salla et al., 2012).

While it is clear that both OTM and obesity mediate the biomarkers in the PDL space, there has been a lack of analysis of biochemical changes with orthodontic treatment of obese subjects. This study investigates obesity-related biomarkers, alongside tissue and bone remodelling biomarkers and inflammation biomarkers, in three human bio-fluids (UWMS, GCF and serum). This is the first comprehensive analysis of biomarker changes during the orthodontic alignment stage of both obese and normal weight patients.

### 5.2.1.1 Aims and objectives

The aim of this prospective cohort study was to investigate the effects of obesity on the level of biomarkers during the alignment stage of orthodontic treatment in obese and normal weight adolescent patients.

To address this aim, the following steps were taken:

1. Biochemical analysis of inflammation, tissue and bone remodelling and obesity-related biomarkers in UWMS, GCF and serum. This analysis was conducted for both normal weight and obese patients, in order to assess which fluid best presents the local biological changes that occur during OTM;
2. Assessment of the effect of orthodontic treatment on the concentrations of selected biomarkers by measuring their levels before the placement of the fixed orthodontic appliance and at three points during their treatment; and

3. Assessment of the effect of obesity on the concentrations of the selected biomarkers by comparing the levels of selected biomarkers between obese and normal weight patients at each time point.

The null hypotheses were that obesity does not modify the biochemical changes that take place during OTM and that there is no difference between UWMS, GCF and serum in the biochemical analysis.

## 5.2.2 Methods

### 5.2.2.1 Sample collection for biochemical analyses

Of the 55 patients recruited for the prospective study, 28 were of normal weight and 27 were obese. UWMS, GCF and serum were collected at four time points, as described in Section 2.4: (T1) start of treatment; (T2) 1 hour following appliance placement; (T3) 1 week following appliance placement; and (T4) end of alignment stage (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch). For all participants, plaque levels and gingival health were measured at T1, T3 and T4, as mentioned in Section 2.3. UWMS and GCF flow rate were measured at T1, T2, T3 and T4 as described in Section 2.6.

Since biochemical changes were more likely to be observed in GCF during OTM, GCF was analysed for all 55 patients (28 normal weight and 27 obese); whilst UWMS and serum were assayed for a random selection of 20 patients (10 normal weight and 10 obese).

UWMS, GCF and serum were analyzed by magnetic Luminex screening assay, using a commercially available kit (R&D systems, Bio-Techne, Abingdon, UK) as mentioned in Section 2.13. The screening was conducted to detect (in pg/ml): adipokines (adiponectin, leptin and resistin), inflammatory mediators (MPO and CRP), the bone remodelling biomarker (RANKL), and tissue remodelling biomarkers (MMP8, MMP9, TIMP1, and their ratios MMP8/TIMP1, MMP9/TIMP1).

### 5.2.2.2 Statistical analysis

Descriptive statistics were used to summarise the outcome variables. Parametric and non-parametric analyses were carried out after checking for the normality distribution using Shapiro-Wilk test of normality. The Friedman test was used to measure the difference in the levels of the not normally distributed data of biomarkers over time for both normal

weight and obese groups. The Mann Whitney U test was used to compare the not normally distributed data of biomarkers' concentrations in normal weight and obese groups at each time point. All statistical analyses were done using SPSS (BM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 23; Armonk; New York; IBM;Corp).

## 5.2.3 Results

### 5.2.3.1 Participants

The clinical parameters of the subjects enrolled in the present study are shown in Table 5.10. Age did not significantly differ between the obese and normal weight groups ($p>0.05$). The BMI was significantly higher in obese group compared to normal weight ($p<0.00001$).

**Table 5.10** Demographics and clinical characteristics of the patients participated in prospective cohort study.

| Sample | Parameters | Normal weight | Obese |
|---|---|---|---|
| **GCF** | Number | 28 | 27 |
| | Age (years): Mean (SD) | 15.1 (1.6) | 15.1 (1.9) |
| | Gender: male / female | 15 / 13 | 12 / 15 |
| | BMI ($kg/m^2$): Mean (SD) | 19.4 (2.2) | 30.2 (3.5) * |
| **UWMS & serum** | Number | 10 | 10 |
| | Age (years): Mean (SD) | 14.9 (1.6) | 15.9 (2.1) |
| | Gender: male / female | 5 / 5 | 3 / 7 |
| | BMI ($kg/m^2$): Mean (SD) | 19.6 (2.3) | 29.7 (2.3) * |

UWMS, unstimulated whole mouth saliva; GCF, gingival crevicular fluid; SD, standard deviation; BMI, body mass index; *,significantly higher ($p<0.00001$) using independent t-test.

### 5.2.3.2 UWMS and GCF flow rate

Both the mechanical stimuli of the orthodontic appliance, and the inflammatory reactions that take place during OTM, may affect the salivary and GCF flow rate.

The Friedman test followed by the Bonferroni correction showed that UWMS and GCF flow rate increased significantly at all-time points (T2-T4) in comparison to the baseline (T1) in both normal weight and obese patients.

Comparing the flow rate between normal weight and obese patients at each time point, the Mann Whitney U test showed that GCF flow rate was significantly higher in

obese patients than in normal weight patients before and throughout the orthodontic alignment, whereas there was no significant difference in UWMS flow rate at any time point (Figure 5.4).

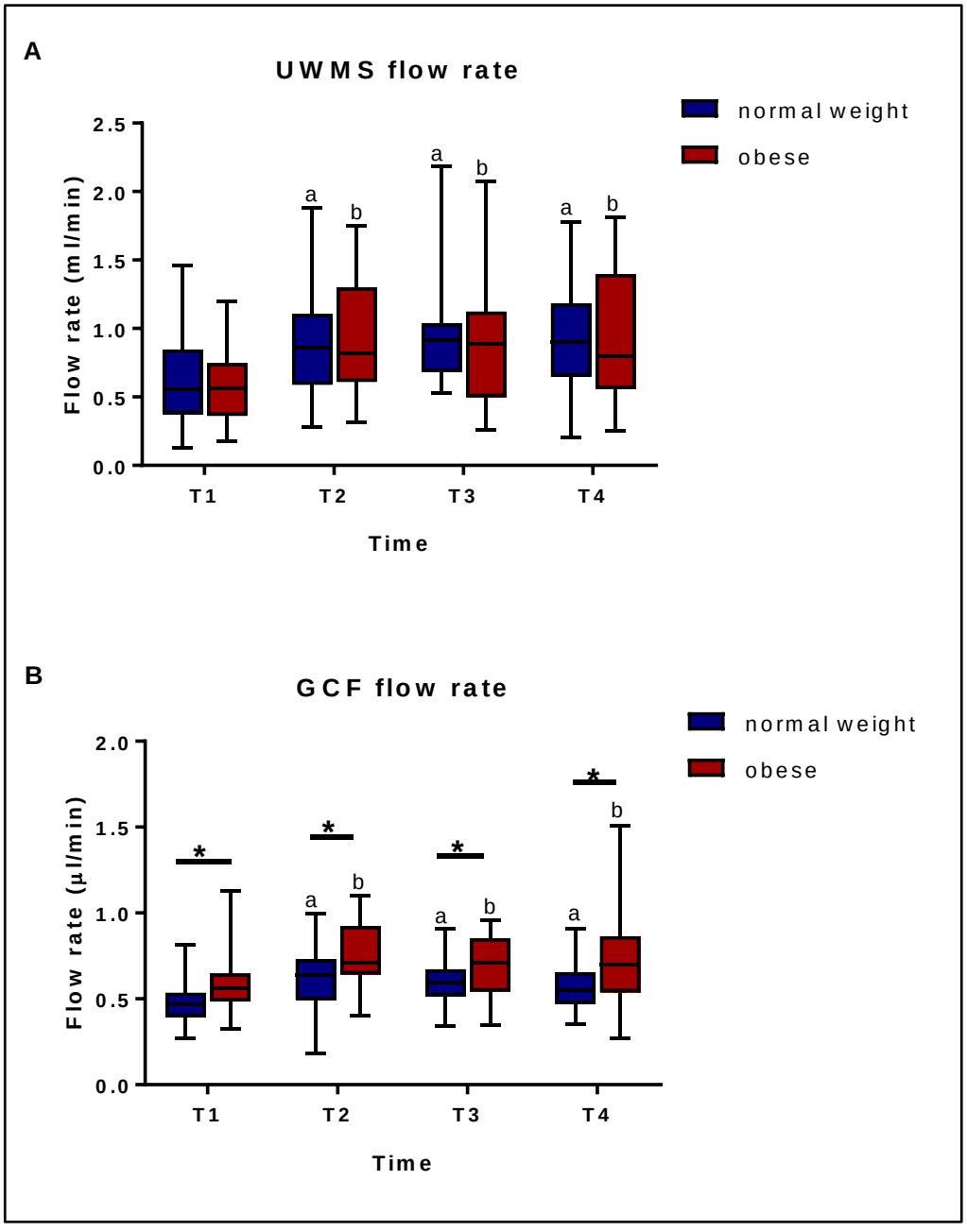



**Figure 5.4** Box plots showing the UWMS, unstimulated whole mouth saliva (A) and GCF, gingival crevicular fluid (B) flow rate of normal weight (n=28) and obese (n=27) patients at four time points. T1, baseline (before placement of orthodontic appliance); T2, 1 hour after placement of orthodontic appliance; T3, 1 week after placement of orthodontic appliance (n=24 in each group); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch); *, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test; (a), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$) for normal weight group; (b), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$) for obese group, vertical whiskers indicate minimum to maximum values.

#### 5.2.3.3 Periodontal health assessment

As bacterial plaque and inflamed gingivae can influence the levels of biomarkers in GCF, a periodontal health measurement is required. The Friedman test, followed by the Bonferroni correction, showed that the plaque index increased significantly in both normal weight and obese patients at the end of alignment (T4), compared with the baseline measurement (T1). However, gingival index significantly increased at T4 in normal weight patients and after 1 week (T3) in obese patients, continuing until the end of alignment. The Mann-Whitney U test showed comparable plaque and gingival scores between the normal weight and obese groups at all-time points (Figure 5.5).

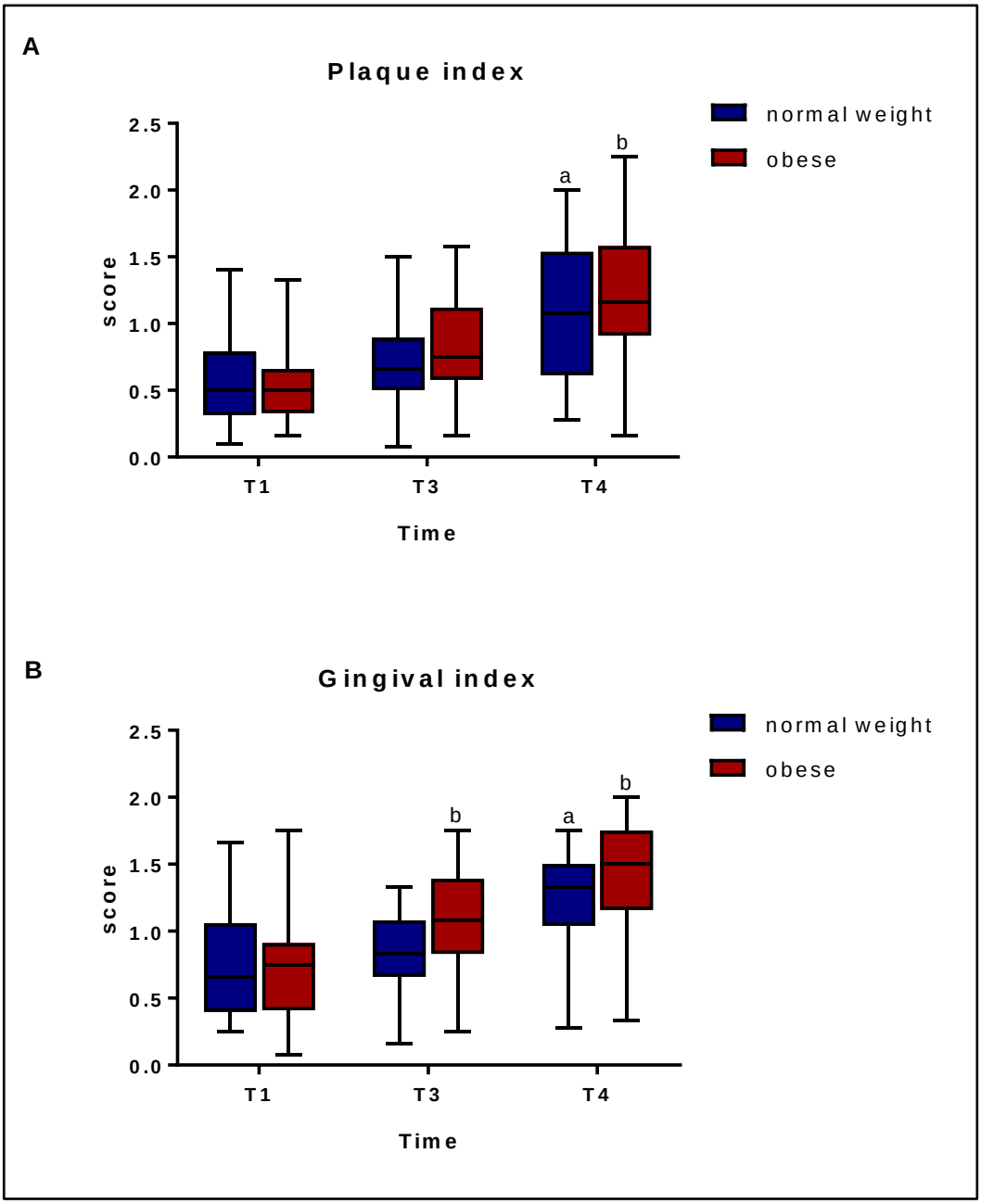



**Figure 5.5** Box plots showing the plaque index (A) and the gingival index (B) of normal weight (n=28) and obese (n=27) patients at four time points. T1, baseline (before placement of orthodontic appliance); T3, 1 week after placement of orthodontic appliance (n=24 in each group); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch); (a), significant difference with T1 using Friedman test followed by Bonferroni correction ($p<0.0166$) for normal weight group; (b), significant difference with T1 using Friedman test followed by Bonferroni correction ($p<0.0166$) for obese group, vertical whiskers indicate minimum to maximum values.

### 5.2.3.4 Biochemical analyses of UWMS

The biochemical analyses of UWMS collected at four time points from 10 normal weight and 10 obese patients showed very few significant changes (Figures 5.6 - 5.10).

Between time points, the only significant biochemical changes in UWMS were observed in the levels of MPO, the biomarker of inflammation (Figure 5.9). MPO levels were significantly increased in obese patients 1 hour after appliance placement (T2) and in normal weight patients after 1 week (T3), compared with baseline levels and continue until the end of alignment. No other biomarkers showed changes over time in UWMS; however, several biomarkers showed significantly higher levels in obese patients compared to normal weight patients at certain time points, including MPO levels at T2 and T3, resistin levels at T2, and TIMP1 at T4 (end of the alignment); whilst, RANKL levels were significantly lower in obese compared to normal weight group at T4.

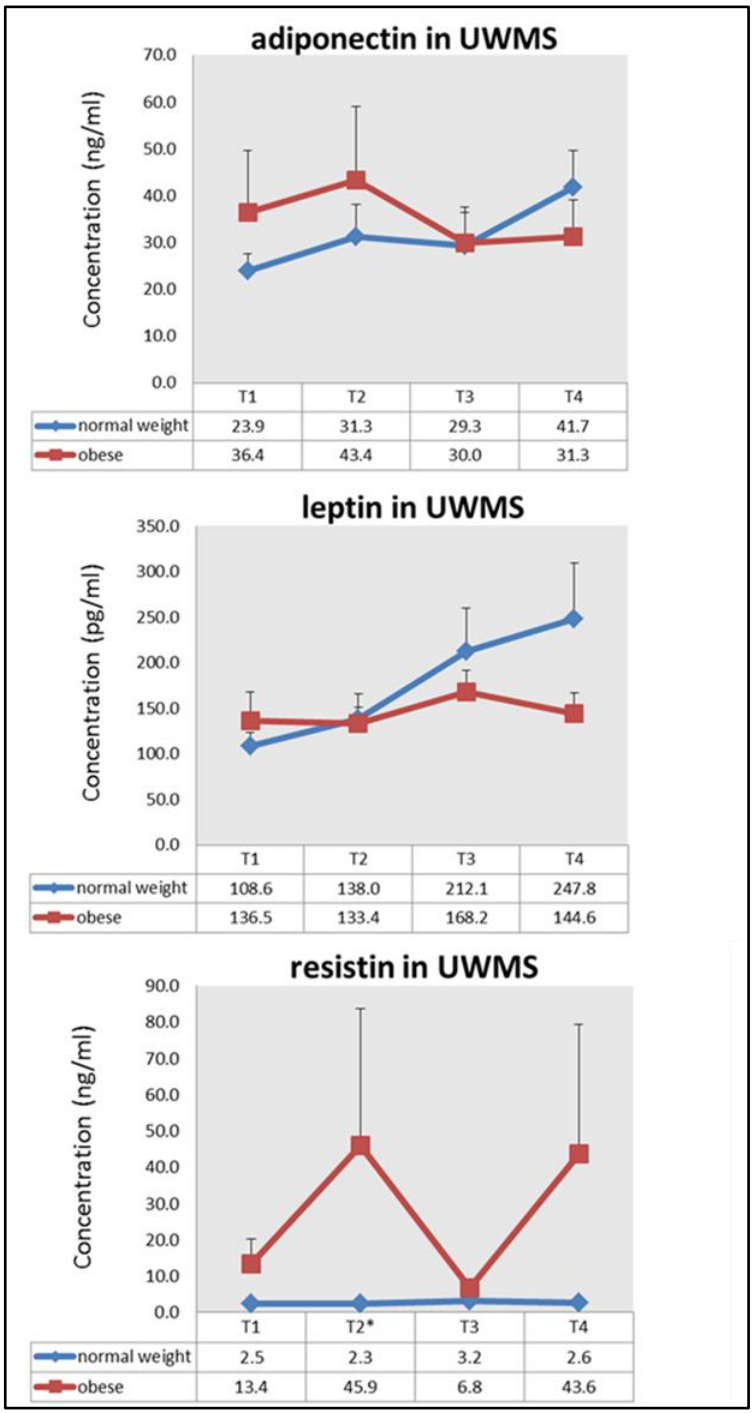



**Figure 5.6** Graphs showing the levels of obesity biomarkers in unstimulated whole mouth saliva (UWMS) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=10); *, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test.

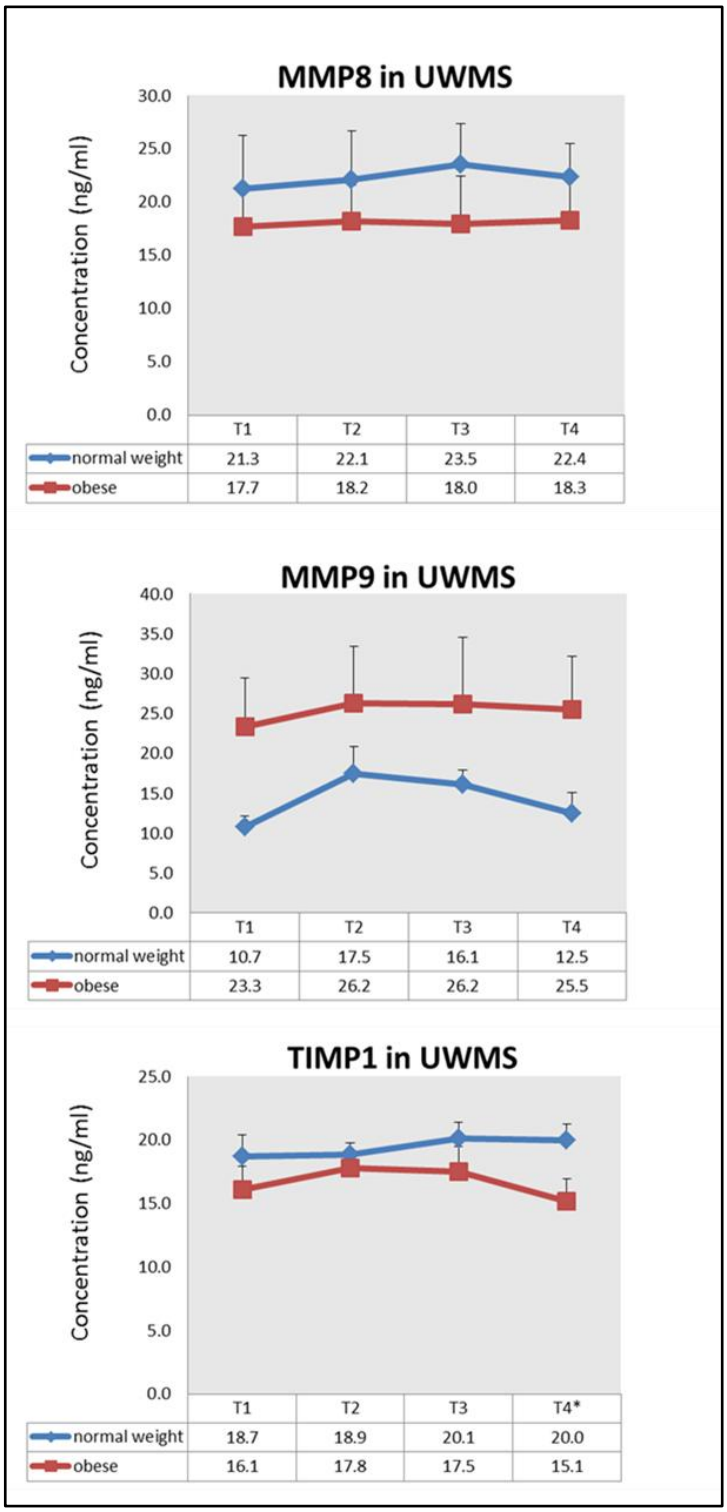



**Figure 5.7** Graphs showing the levels of tissue remodelling biomarkers in unstimulated whole mouth saliva (UWMS) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=10); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; *, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test.

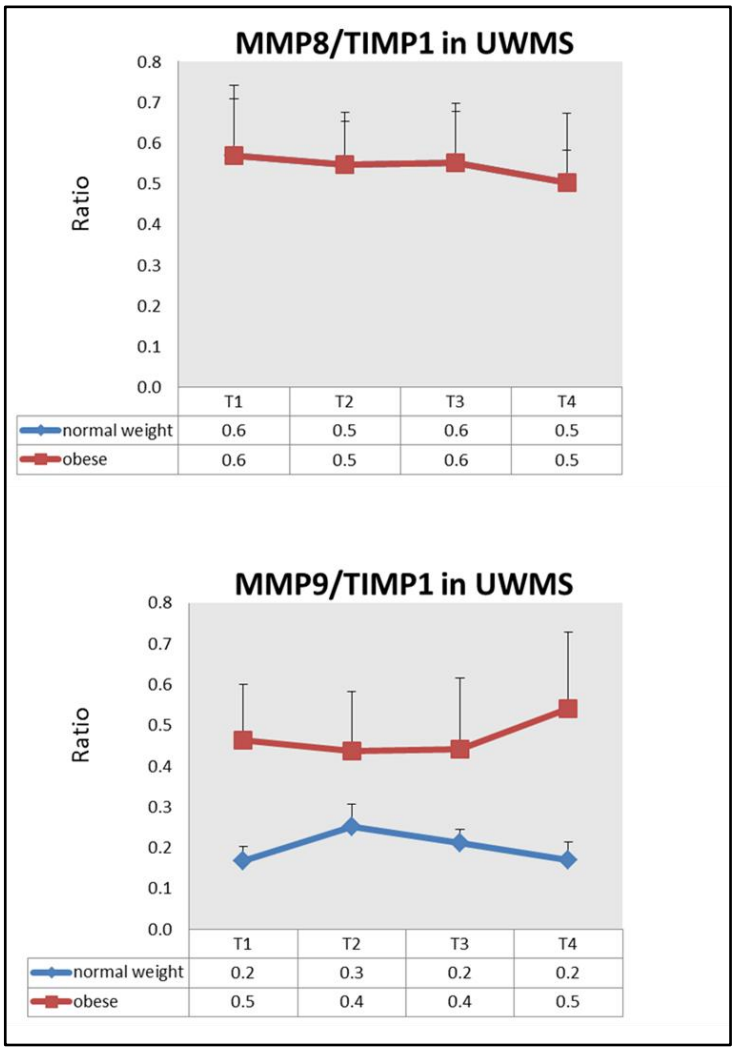



**Figure 5.8** Graphs showing the levels of MMP8/TIMP1 and MMP9/TIMP1 ratios in unstimulated whole mouth saliva (UWMS) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=10); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1.

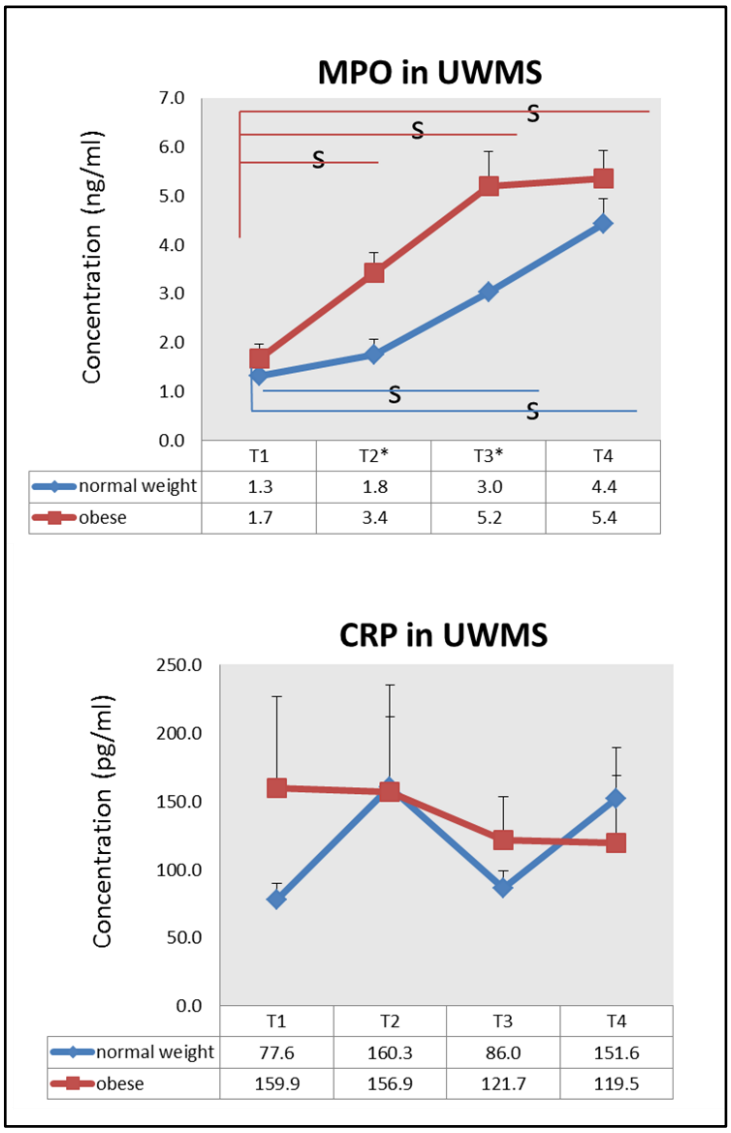



**Figure 5.9** Graphs showing the levels of inflammation biomarkers in unstimulated whole mouth saliva (UWMS) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=10); MPO, myeloperoxidase; CRP, C reactive protein; (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$); *, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test.

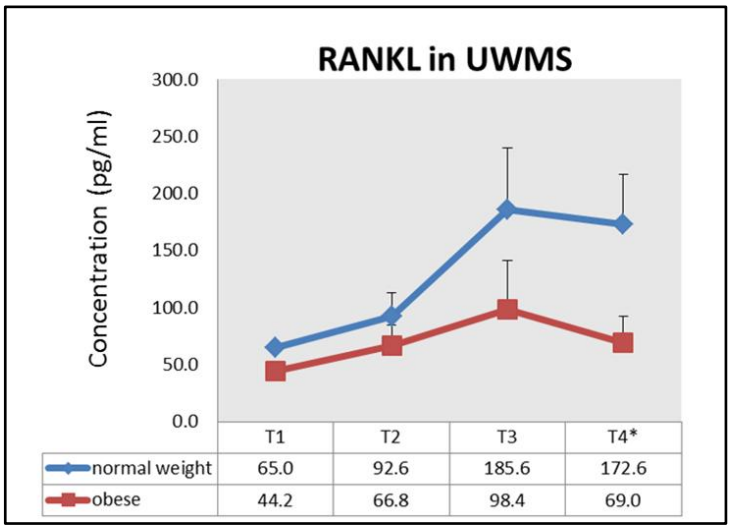



**Figure 5.10** Graph showing the levels of bone remodelling biomarkers (RANKL) in unstimulated whole mouth saliva (UWMS) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=10); RANKL, receptor activator of nuclear factor kappa-B ligand;*, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test.

### 5.2.3.5 Biochemical analyses of serum

The biochemical analyses of serum collected at four time points from 10 normal weight and 10 obese patients showed no changes in the level of inflammatory biomarkers (MPO and CRP) during the orthodontic alignment (Figure 5.11). Only the levels of adiponectin among the obesity biomarkers were significantly reduced in both normal weight and obese patients at the end of alignment (T4) (Figure 5.12). In contrast, the levels of RANKL (bone remodelling biomarkers) were significantly increased at T4 in both normal weight and obese groups (Figure 5.13). Among the tissue remodelling biomarkers, the levels of MMP8 significantly increased, while TIMP1 levels significantly reduced by the end of alignment (T4) in obese patients only (Figure 5.14). Such changes result in significant increase in the MMP8/TIMP1 ratio in the same group over time (T2-T4) (Figure 5.15).

Comparing the levels of circulating biomarkers between normal weight and obese patients at each time point showed that, among the inflammatory biomarkers (MPO and CRP), comparable levels of MPO were observed between the two groups. However, the levels of circulating CRP were significantly higher in obese patients before and throughout the orthodontic alignment (Figure 5.11). The same findings were observed for leptin (obesity biomarker) levels, which were higher in obese than in normal weight patients, from the baseline until the end of alignment (T1-T4). Conversely, the circulating levels of the other obesity biomarker, adiponectin, were significantly reduced in obese patients at the baseline (T1) and at the end of alignment (T4). The levels of the third obesity biomarker, resistin, did not significantly change at all time points in both groups (Figure 5.12). The levels of RANKL (bone remodelling biomarker) were significantly increased in the serum of obese patients compared to normal weight patients at the end of alignment (T4) (Figure 5.13). Similarly, the tissue remodelling biomarkers showed minor changes at the end of the alignment (T4), including higher levels of circulating MMP8 and lower levels of TIMP1 in obese patients (Figure 5.14). Therefore, a significantly higher MMP8/TIMP1 ratio was observed in obese patients at T4, in comparison with normal weight patients (Figure 5.15).

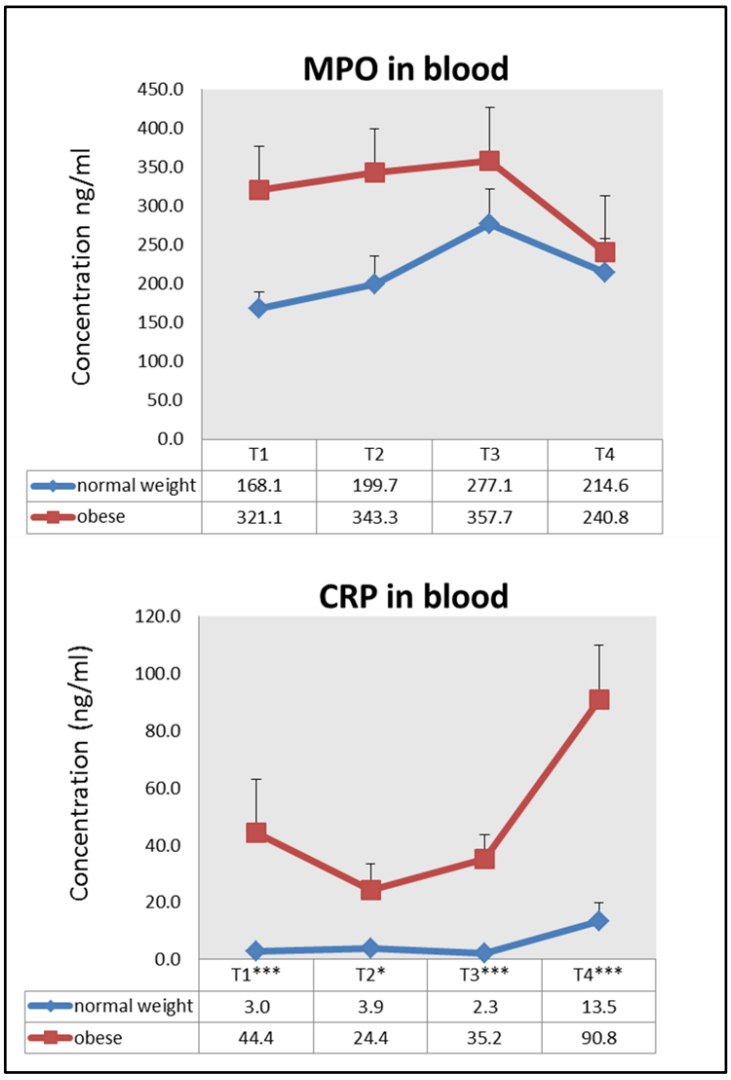



**Figure 5.11** Graphs showing the levels of inflammation biomarkers in serum of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=10); MPO, myeloperoxidase; CRP, C reactive protein; *, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test; ***, significant difference between normal weight and obese ($p<0.001$) by Mann Whitney U test.

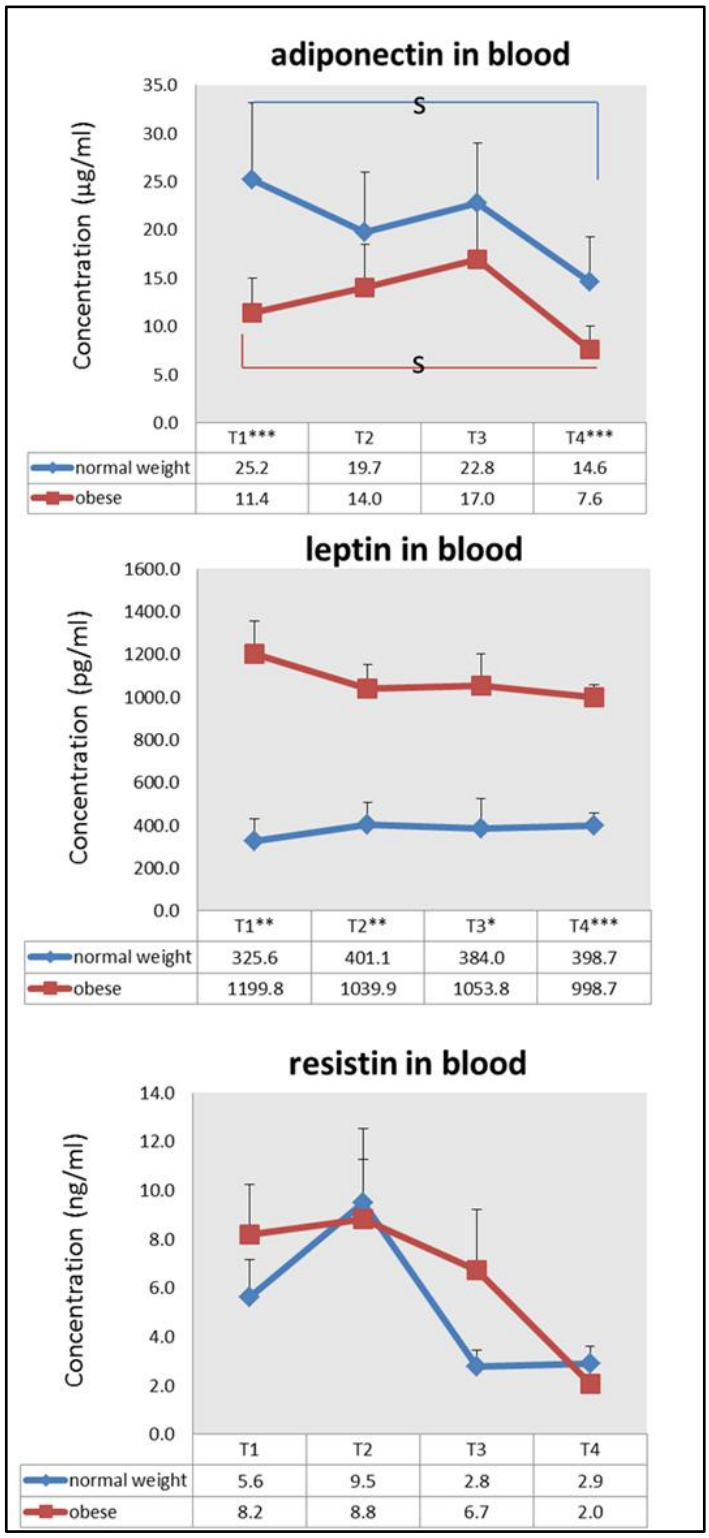



**Figure 5.12** Graphs showing the levels of obesity biomarkers in serum of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch (n=10); (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$);*, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test; **, significant difference between normal weight and obese ($p<0.01$) by Mann Whitney U test; ***, significant difference between normal weight and obese ($p<0.001$) by Mann Whitney U test.

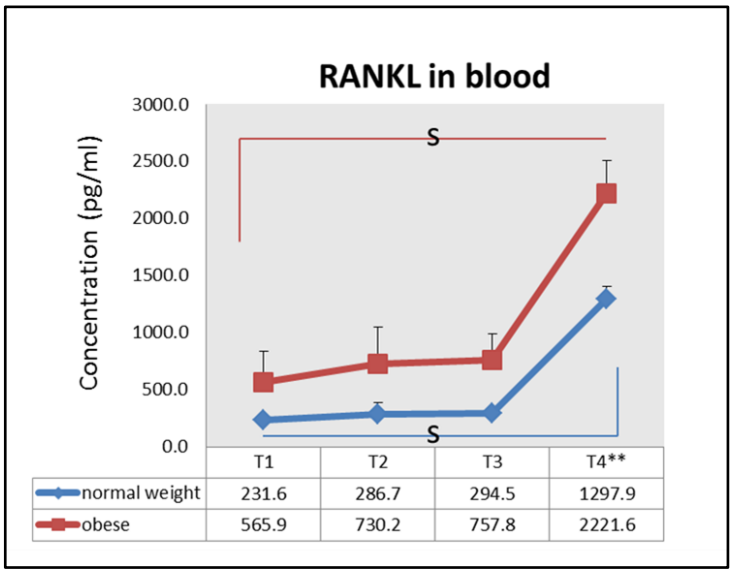



**Figure 5.13** Graph showing the levels of bone remodelling biomarkers (RANKL) in serum of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=10); RANKL, receptor activator of nuclear factor kappa-B ligand; (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$); **, significant difference between normal weight and obese ($p<0.01$) by Mann Whitney U test.

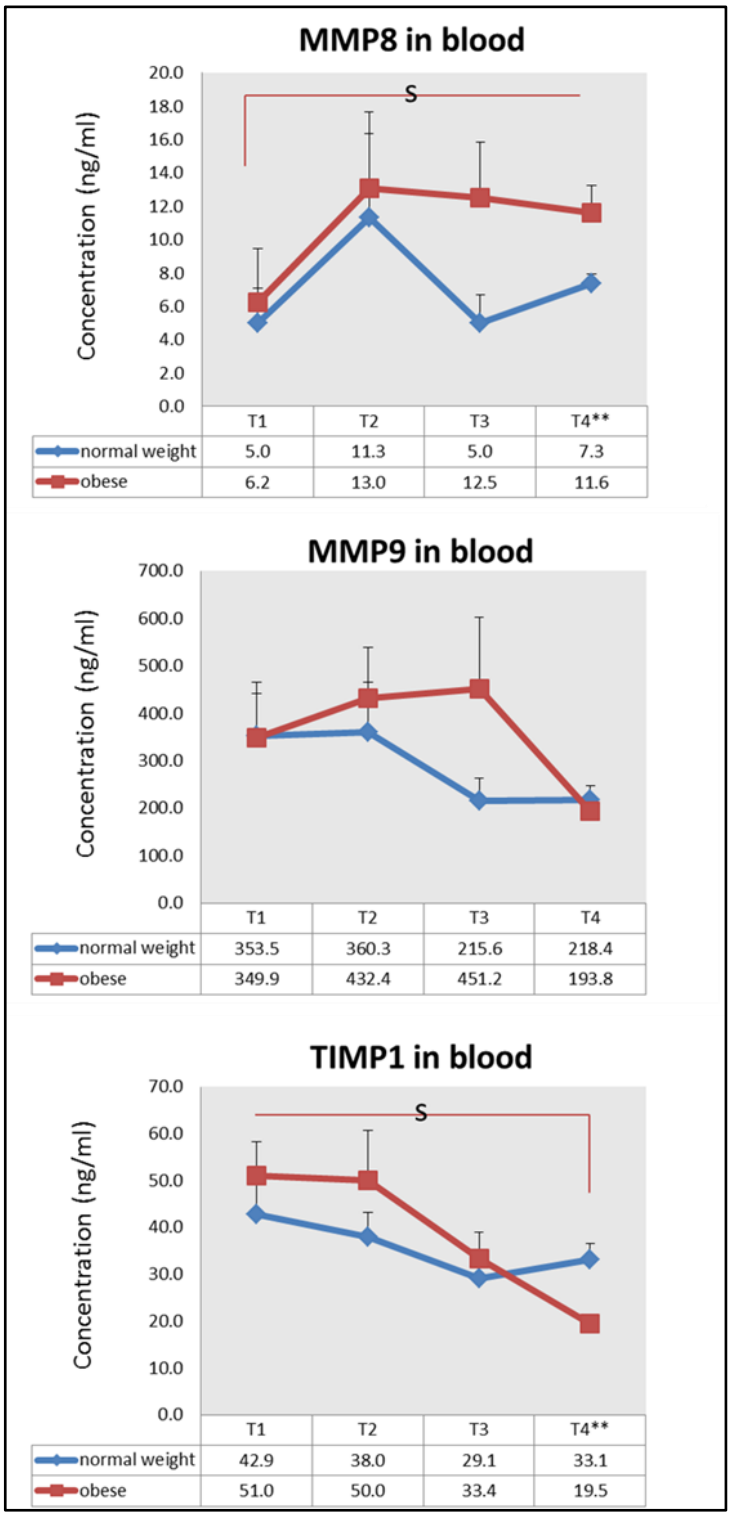



**Figure 5.14** Graphs showing the levels of tissue remodelling biomarkers in serum of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch (n=10); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$); **, significant difference between normal weight and obese ($p<0.01$) by Mann Whitney U test.

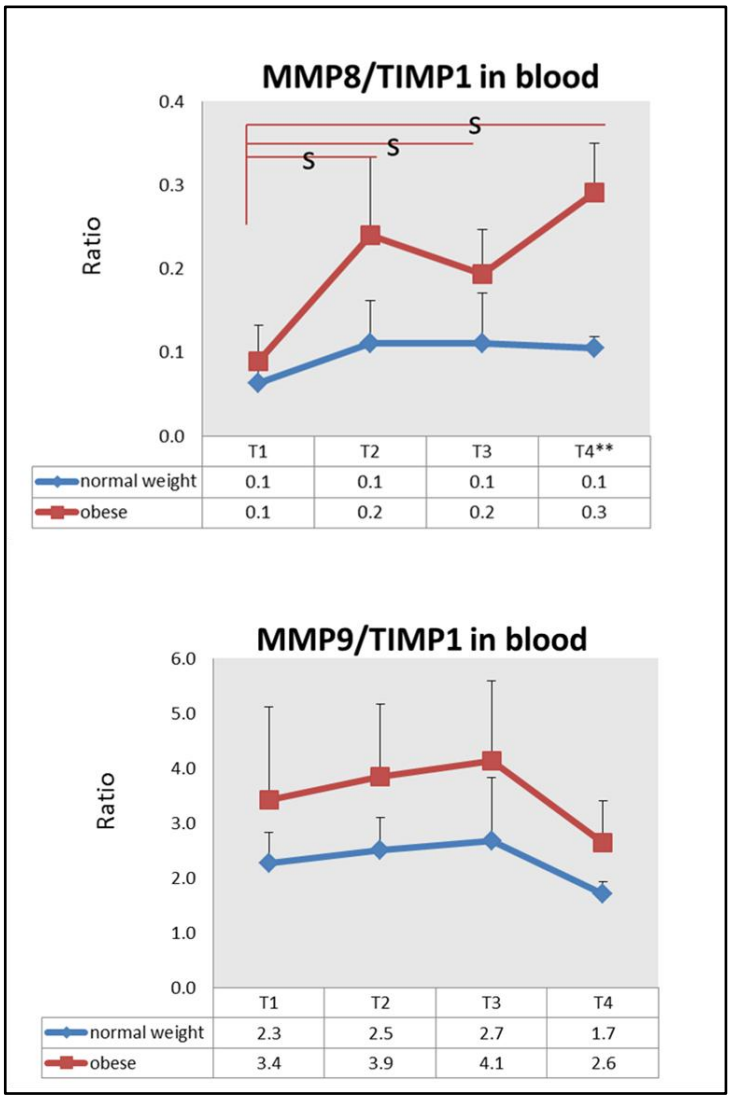



**Figure 5.15** Graphs showing the levels of MMP8/TIMP1 and MMP9/TIMP1 ratios in serum at four time points of normal weight and obese patients. T1, baseline (before placement of orthodontic appliance) (n=10); T2, 1 hour after placement of orthodontic appliance (n=10); T3, 1 week after placement of orthodontic appliance (n=8); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=10); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$); **, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test.

### 5.2.3.6 Biochemical analyses of GCF

The results of our cross-sectional study, alongside the findings from the literature review, demonstrate that GCF is more likely to be changed by the biological reactions that take place in the periodontal tissues during OTM. GCF from all 55 patients was therefore analysed in this prospective study. The biochemical analyses of GCF collected from 28 normal weight and 27 obese patients at four time points showed more changes than those seen in UWMS and serum during the orthodontic alignment stage.

No change in the level of obesity biomarkers (adiponectin, leptin and resistin) was observed at all-time points of orthodontic alignment (Figure 5.16).

Tissue remodelling biomarkers (MMP8, MMP9, and TIMP1) showed that MMP8 and TIMP1 levels increased only in the normal weight group. MMP8 increased after 1 hour (T2), whereas TIMP1 increased after 1 week until the end of the alignment (T3-T4). Conversely, the levels of MMP9 were significantly increased at T2 and T3 in both normal weight and obese groups, then returned to the baseline level at T4 (Figure 5.17). The ratios of MMP8/TIMP1 and MMP9/TIMP1 therefore changed accordingly. The MMP8/TIMP1 ratio increased at T4 in the normal weight group, while the MMP9/TIMP1 ratio increased at T2 and T4 in the normal weight group and at T2 in obese patients (Figure 5.18).

Analysis of the inflammatory biomarkers (MPO and CRP) showed that the levels of MPO were significantly increased 1 hour after appliance placement (T2) in obese patients and after 1 week (T3) in normal weight patients until the end of alignment. CRP levels showed no changes in GCF during the orthodontic alignment in both groups (Figure 5.19).

The levels of bone remodelling biomarker (RANKL) were significantly increased at the end of the alignment (T4) in both normal weight and obese patients (Figure 5.20).

Comparing the levels of biomarkers between normal weight and obese patients at each time point showed that the levels of some biomarkers were significantly higher in the obese group at baseline (T1) and continued to remain higher throughout the study period such as MMP8, TIMP1, RANKL, MPO, leptin and resistin. Other biomarkers, such as adiponectin, showed comparable levels in both normal weight and obese groups from baseline to the end of the alignment stage (T1-T4). MMP9 levels were higher in obese patients after 1 hour of appliance placement (T2), then returned to the same levels as for normal weight patients at T3 and T4, whereas CRP levels were higher in obese patients after 1 week of appliance placement (T3) and continued until the end of alignment (T4).

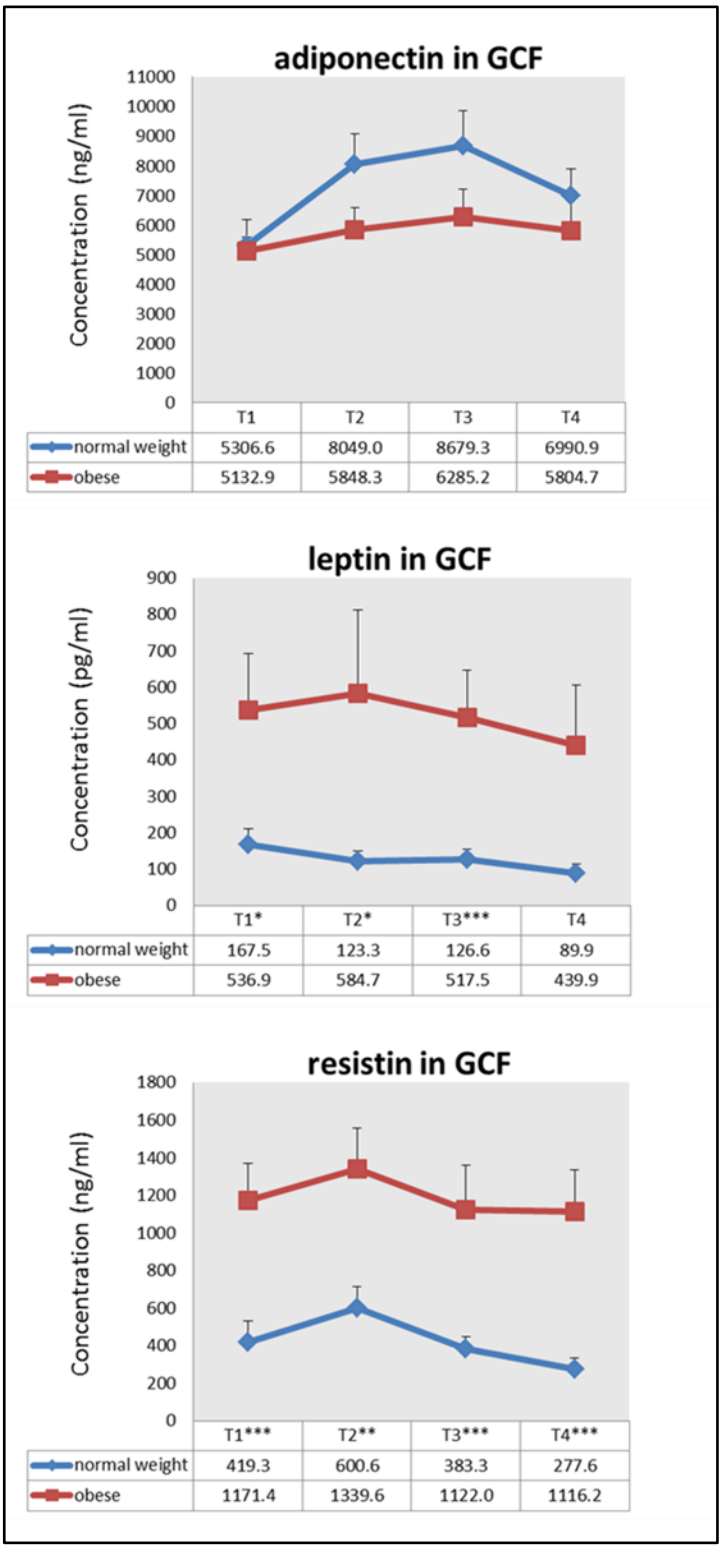



**Figure 5.16** Graphs showing the levels of obesity biomarkers in gingival crevicular fluid (GCF) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=28 normal weight and 27 obese); T2, 1 hour after placement of orthodontic appliance (n=28 normal weight and 27 obese); T3, 1 week after placement of orthodontic appliance (n=24 normal weight and 24 obese); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=28 normal weight and 27 obese); *, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test; **, significant difference between normal weight and obese ($p<0.01$) by Mann Whitney U test, ***: significant difference between normal weight and obese ($p<0.001$) by Mann Whitney U test.

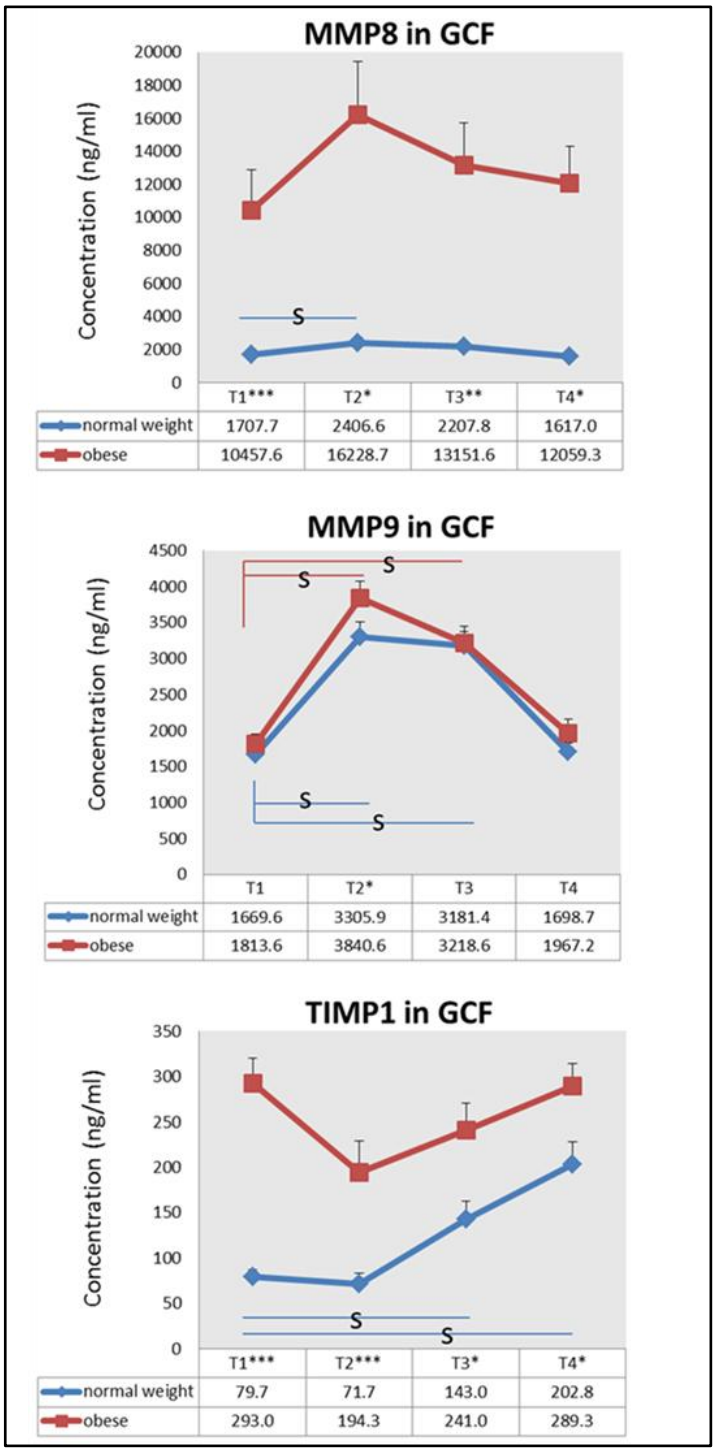



**Figure 5.17** Graphs showing the levels of tissue remodelling biomarkers in gingival crevicular fluid (GCF) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=28 normal weight and 27 obese); T2, 1 hour after placement of orthodontic appliance (n=28 normal weight and 27 obese); T3, 1 week after placement of orthodontic appliance (n=24 normal weight and 24 obese); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=28 normal weight and 27 obese); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$);*, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test; **, significant difference between normal weight and obese ($p<0.01$) by Mann Whitney U test; ***, significant difference between normal weight and obese ($p<0.001$) by Mann Whitney U test.

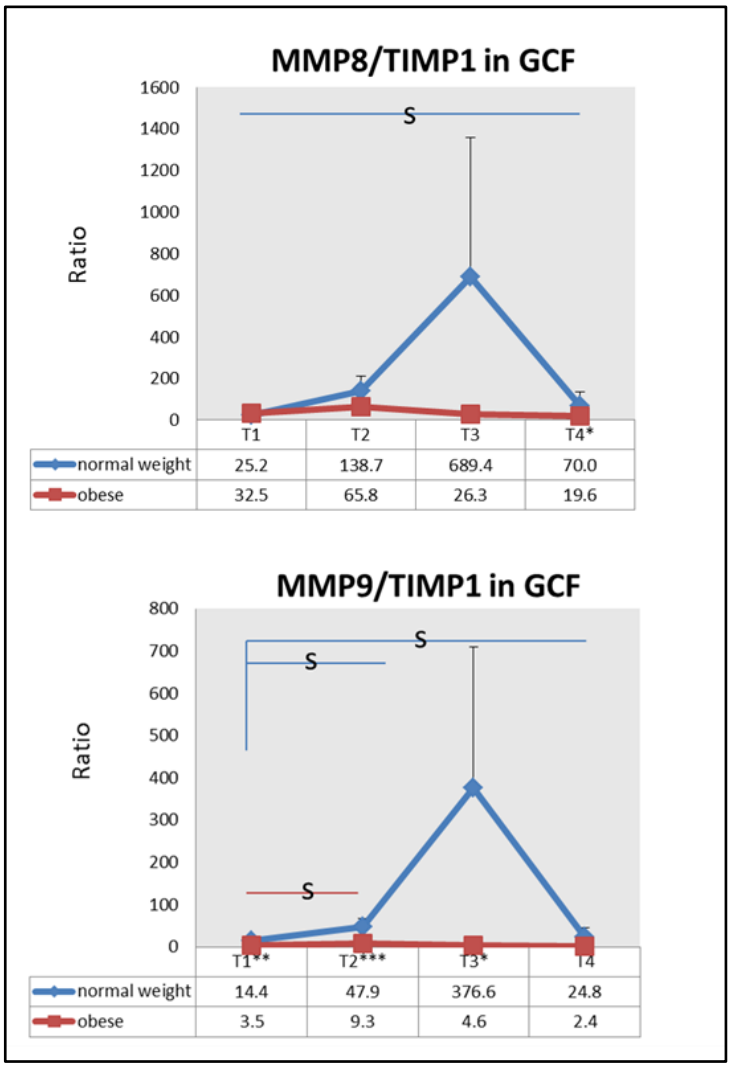



**Figure 5.18** Graphs showing the levels of MMP8/TIMP1 and MMP9/TIMP1 ratios in gingival crevicular fluid (GCF) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=28 normal weight and 27 obese); T2, 1 hour after placement of orthodontic appliance (n=28 normal weight and 27 obese); T3, 1 week after placement of orthodontic appliance (n=24 normal weight and 24 obese); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=28 normal weight and 27 obese); MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$);*, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test; **, significant difference between normal weight and obese ($p<0.01$) by Mann Whitney U test; ***, significant difference between normal weight and obese ($p<0.001$) by Mann Whitney U test.

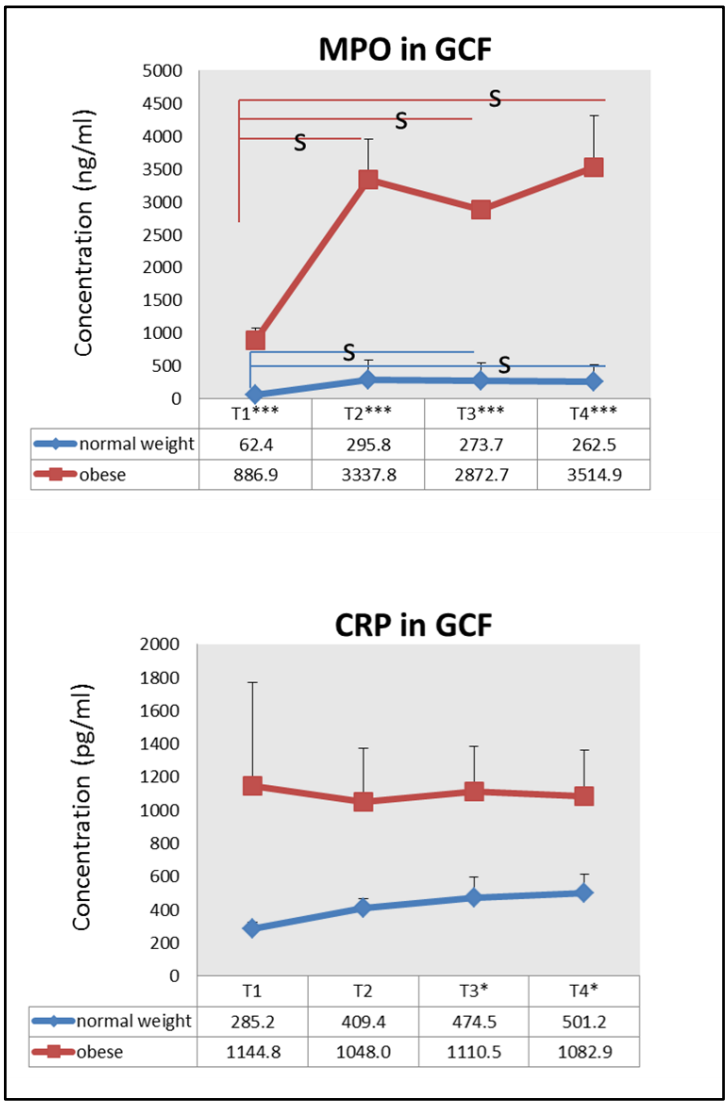



**Figure 5.19** Graphs showing the levels of inflammation biomarkers in gingival crevicular fluid (GCF) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=28 normal weight and 27 obese); T2, 1 hour after placement of orthodontic appliance (n=28 normal weight and 27 obese); T3, 1 week after placement of orthodontic appliance (n=24 normal weight and 24 obese); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=28 normal weight and 27 obese); MPO, myeloperoxidase; CRP, C reactive protein; (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$);*, significant difference between normal weight and obese ($p<0.05$) by Mann Whitney U test.; ***, significant difference between normal weight and obese ($p<0.001$) by Mann Whitney U test.

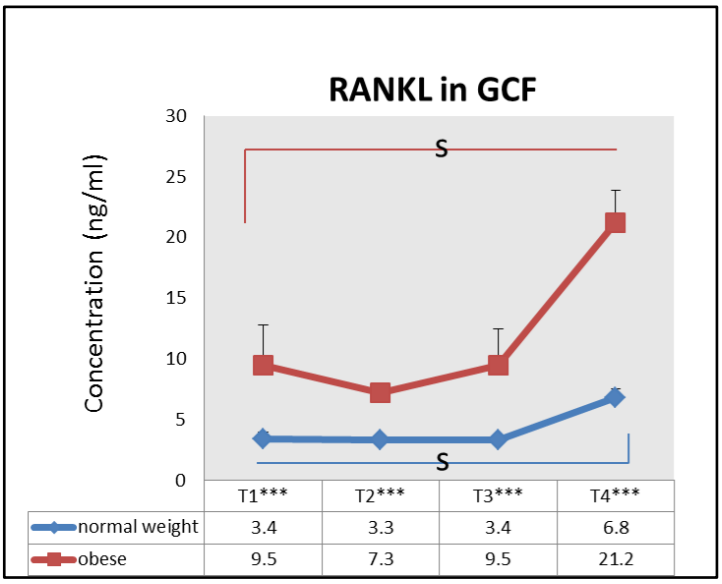



**Figure 5.20** Graph showing the levels of bone remodelling biomarkers (RANKL) in gingival crevicular fluid (GCF) of normal weight and obese patients at four time points. T1, baseline (before placement of orthodontic appliance) (n=28 normal weight and 27 obese); T2, 1 hour after placement of orthodontic appliance (n=28 normal weight and 27 obese); T3, 1 week after placement of orthodontic appliance (n=24 normal weight and 24 obese); T4, end of the alignment (0.019 x 0.025-inch stainless steel rectangular archwire in the lower arch) (n=28 normal weight and 27 obese); RANKL, receptor activator of nuclear factor kappa-B ligand; (s), significant difference with T1 by Friedman test followed by Bonferroni correction ($p<0.0125$); ***, significant difference between normal weight and obese ($p<0.001$) by Mann Whitney U test.

### 5.2.3.7 Correlation of biomarkers with periodontal health

Since bacterial plaque and inflamed gingivae can potentially influence the GCF flow rate and the production of some biomarkers, regression analysis was performed of the outcome (the GCF flow rate and biomarker levels), the explorative factor (plaque index or gingival index) and the variation through time points. The results showed that there was no significant correlation between the GCF flow rate and the levels of biomarkers with plaque and gingival indices (Table 5.11).

**Table 5.11** Results of regression models assessing the effect of PI and GI on the levels of biomarkers in prospective cohort study.

| | **PI (transformed)** | | **GI (transformed)** | |
|---|---|---|---|---|
| GCF parameters (Log10 transformed) | Coefficient (95% CI) | P value | Coefficient (95% CI) | P value |
| GCF flow rate | 0.06 (-0.04,0.15) | 0.248 | 0.08 (-0.02,0.18) | 0.117 |
| Adiponectin (pg/ml) | -0.11 (-0.35,0.13) | 0.353 | -0.06 (-0.33,0.21) | 0.671 |
| Leptin‡ (pg/ml) | 2.31 (-4.97,9.59) | 0.534 | 3.23 (-5.24,11.70) | 0.455 |
| Resistin (pg/ml) | -0.11 (-0.37,0.15) | 0.423 | 0.13 (-0.22,0.49) | 0.461 |
| MPO (pg/ml) | -0.16 (-0.61,0.30) | 0.505 | -0.08 (-0.60,0.43) | 0.749 |
| CRP† (pg/ml) | 0.03 (-0.24,0.29) | 0.852 | 0.01 (-0.36,0.37) | 0.975 |
| MMP8 (pg/ml) | 0.18 (-0.09,0.45) | 0.189 | 0.17 (-0.11,0.45) | 0.239 |
| MMP9† (pg/ml) | 0.18 (-0.06,0.42) | 0.148 | 0.14 (-0.14,0.42) | 0.338 |
| TIMP1 (pg/ml) | 0.07 (-0.14,0.28) | 0.505 | 0.14 (-0.13,0.40) | 0.315 |
| MMP8/TIMP1 (pg/ml) | 0.13 (-0.17,0.44) | 0.402 | 0.12 (-0.23,0.47) | 0.500 |
| MMP9/TIMP1 (pg/ml) | 0.14 (-0.14,0.42) | 0.333 | 0.08 (-0.27,0.42) | 0.667 |
| RANKL† (pg/ml) | -0.01 (-0.24,0.22) | 0.934 | 0.05 (-0.22,0.32) | 0.722 |

PI, plaque index; GI, gingival index; GCF, gingival crevicular fluid; MPO, myeloperoxidase; CRP, C reactive protein; MMP8, matrix metalloproteinase-8; MMP9, matrix metalloproteinase-9; TIMP1, tissue inhibitor of metalloproteinase 1; RANKL, receptor activator of nuclear factor kappa-B ligand. †Log10 transformation improved the skewness of the data, but the Shapiro-Wilk test indicated that transformed data were still not normally distributed. Therefore, the median (interquartile range) is presented instead of mean (SD) and the Mann-Whitney test is used on the transformed data instead of the unpaired t-test. ‡ Square root transformation used instead of log10, as several null values were included. Therefore, the median (interquartile range) is presented instead of mean (SD) and the Mann-Whitney test is used on the transformed data instead of the unpaired t-test.

## 5.2.4 Discussion

In OTM, the periodontium endures an aseptic inflammation, in which various cytokines are released by the effect of mechanical forces (Salla et al., 2012). This study was designed to assess the effect of obesity on the level of inflammation, tissue and bone remodelling, and obesity-related biomarkers in UWMS, GCF and serum before and during the alignment stage of fixed appliance orthodontic treatment. Four time points were chosen in an attempt to take into account the different biological mechanisms characterizing the initial, lag and postlag phases of OTM. The study evaluated the levels of biomarkers compared with the baseline measurement (T1) and described the difference in the levels of selected biomarkers between normal weight and obese patients at each of the four time points.

### 5.2.4.1 UWMS and GCF flow rate

The UWMS flow rate increased significantly after the placement of the fixed orthodontic appliance, with no significant differences between normal weight and obese groups, both before and throughout the orthodontic alignment. This indicates that the cause of more saliva production is the mechanical stimulation by orthodontic appliance, rather than patient BMI. The findings are in agreement with data collected in previous salivary flow rate studies, indicating no difference between obese and healthy controls (Powers et al., 1981, Epstein et al., 1996). On the contrary, reduced whole saliva was observed in adults aged <50 years with BMI>25 (Flink et al., 2008). Reduced stimulated whole saliva is also an observation from childhood obesity studies (Modéer et al., 2010). They attribute the reduced salivary flow to the storage of adipocytesin in the parotid gland, in addition to increased pro-inflammatory cytokines that may affect the function of the salivary glands. These controversial findings may result from differences in sample size, patient age, and the method and time of sample collection. The limitation of our study is that samples were collected at various times during the day and therefore, to some extent, the salivary flow rate results may be influenced by circadian rhythms (Flink et al., 2008).

Previous studies have suggested that GCF flow reflects changes in deeper periodontal tissues, such as the alveolar bone and the PDL of the teeth, while under the influence of orthodontic treatment (Grieve et al., 1994, Heasman et al., 1996, Iwasaki et al., 2005, Iwasaki et al., 2001, Tuncer et al., 2005, Krishnan and Davidovitch, 2006b, Masella and Meister, 2006). Since there was no association between GCF flow rates and plaque and

gingival scores, the increased GCF flow rate resulted from the influence of both obesity and fixed appliance orthodontic treatment. The effect of obesity can be seen in the higher GCF flow rate in obese patients before and throughout all time points of orthodontic alignment. This may be related to the inflammatory effects of obesity on the periodontal tissues that impacts on the permeability of blood vessels and GCF flow rate in obese subjects. This has been confirmed by previous studies highlighted that inflammation can modify the rate and composition of GCF (Kavadia-Tsatala et al., 2001).

The significant increase of GCF flow rate after orthodontic appliance placement may indicate the effect of orthodontic treatment. These findings are confirmed by previous studies that demonstrate the impact of orthodontic forces on the volume of GCF (Basaran et al., 2006, Karacay et al., 2007); however, these findings are not consistent, as some studies did not demonstrate a significant influence of mechanical stress on GCF flow (Apajalahti et al., 2003, Ingman et al., 2005, Bildt et al., 2009). One explanation for this difference might derive from the different methods used for GCF collection.

GCF volume is not necessarily influenced by OTM, but instead is influenced by inflammation of the periodontal tissue (Nishijima et al., 2006, Perinetti et al., 2002). Our results confirm this, as the GCF flow rate was higher as a consequence of both obesity-related inflammation and the inflammation that occurs as a result of orthodontic treatment.

#### 5.2.4.1 Biochemical analyses of UWMS

Significant changes in salivary biomarker levels were restricted to the inflammatory mediator, MPO. The MPO levels in UWMS reflect, to a certain extent, the levels of MPO in GCF, indicating an increase in the number of PMNs in the periodontal tissue due to inflammation from the orthodontic treatment. These findings are in agreement with the results of a previous study, showing a significant increase of MPO in UWMS and GCF 2 hours after orthodontic force application (Marcaccini et al., 2010). The same results were observed in another study using different amount of force (Navarro-Palacios et al., 2014). In our study, however, MPO levels were significantly increased after force application at 1 hour (T2) in obese patients and 1 week (T3) in normal weight patients. The differences between the two groups could be related to the increased recruitment and activation of PMNs in consistence with the inflammatory status associated with obesity.

The significantly higher MPO levels in obese patients, compared with normal weight patients, at T2 and T3 time points, indicate that obesity influences the immune

system and increases PMNs in periodontal tissues which represent one of the most innate immune system components, displaying potent phagocytic and antimicrobial activity (Nijhuis et al., 2009).

### 5.2.4.2 Biochemical analyses of serum

Significant changes in the level of biomarkers in serum were limited to adiponectin, RANKL, MMP8 and TIMP1 at the end of alignment (T4).

The reduction of adiponectin levels in both normal weight and obese patients may reflect the increased inflammation accompanied with tissue and bone metabolism during orthodontic treatment, irrespective of obesity. This can be confirmed by the findings of studies that report reduced serum adiponectin with inflammation such as periodontitis, independent of BMI (Saito et al., 2008, Furugen et al., 2008, Zimmermann et al., 2013); however, there is no previous studies that measure the levels of adiponectin during orthodontic treatment to provide a direct comparison. Conversely, serum RANKL levels were increased in both normal weight and obese groups at the end of the alignment (T4), which mostly reflects the postlag phase of OTM, in which higher bone remodelling takes place for more tooth movement. This reduction in adiponectin and increase in RANKL levels were significantly greater in obese patients than normal weight, indicating greater inflammation and bone resorption in obese patients comparing to normal weight. The possible reason behind this is the high pro-inflammatory cytokines produced by adipocytes, which suppress the anti-inflammatory function of adiponectin and stimulate the RANKL/RANK pathway, the key to osteoclastogensis and bone resorption. These findings partly agreed with a previous study that measured the levels of OPG, RANKL and adiponectin in the serum of obese and lean subjects, which confirmed reduced levels of adiponectin and unchanged RANKL levels in the serum of obese subjects (Ashley et al., 2011).

The significant increase in MMP8 and decrease in TIMP1 at the end of alignment (T4) in obese patients resulted in a significant increase of the MMP8/TIMP1 ratio at (T2, T3, and T4), without any significant changes in the levels of MMP9. The exact reason for this is unclear, but it possibly relates to higher neutrophil activation (the main source of MMP8) that occurs with obesity. These results are confirmed by a study conducted by Belo and co-workers that demonstrated higher MMP8 (but not MMP9) and lower TIMP1 in obese groups, compared to non-obese children and adolescents (Belo et al., 2009).

The circulating levels of other biomarkers, such as MPO and resistin, did not show any significant changes in both normal weight and obese groups. Others, such as leptin and CRP, did not change with orthodontic treatment, although their levels were already significantly higher in obese patients before and throughout the alignment stage. These findings can be related to the role of leptin in the host defence mechanism against infection (Liu et al., 2005) and in response to pro-inflammatory stimuli such as LPS and TNF-α, which increase with obesity (Karthikeyan and Pradeep, 2007a, Shimada et al., 2010). Similarly, high levels of CRP are associated with the inflammatory conditions of obesity (Ellulu et al., 2016) and higher production of IL-6 by adipocytes that influence the production of CRP by hepatocytes to blood (Berg and Scherer, 2005, Nibali et al., 2007). Furthermore, the comparable levels of leptin and CRP at all-time points comparing to baseline indicate that orthodontic treatment results in more local, rather than systemic, inflammation.

### 5.2.4.3 Biochemical analyses of GCF

The results from the regression models did not demonstrate any significant association between plaque and gingival indices and the levels of biomarkers at all-time points. This indicates that the variation in the levels of biomarkers in GCF should result from the response of periodontal tissues to the applied orthodontic force.

This study outlined that obesity-related biomarkers, with both anti-inflammatory (adiponectin) and pro-inflammatory (leptin and resistin) effects, did not significantly change in GCF during orthodontic treatment for both normal weight and obese patients. These findings indicated that orthodontic force did not influence the levels of adipokines in GCF. This result disagrees with a previous study, which reported reduced levels of GCF leptin during orthodontic treatment (Dilsiz et al., 2010); however, the relationship between GCF resistin and adiponectin with OTM has not previously been investigated. In relation to periodontitis, existing literature reports reduced levels of leptin in GCF with periodontal diseases (Karthikeyan and Pradeep, 2007a), whilst the GCF resistin levels were reduced (Zimmermann et al., 2013) or increased with periodontitis (Patel and Raju, 2014) and/or obesity (Suresh et al., 2016). Moreover, in this study, leptin and resistin proteins were higher in GCF of obese patients than in normal weight patients before the placement of the orthodontic appliance and throughout the whole orthodontic alignment. This can be confirmed by a correlation between leptin (Considine et al., 1996) and resistin (Patel and

Raju, 2014) with BMI, and may explain the correlation between leptin and resistin with the high rate of OTM in obese patients, as mentioned in Section 5.1 of this study. The high levels of leptin and resistin associated with obesity may result from the increased size and number of adipocytes, in addition to the release of these proteins by peripheral mononuclear cells and macrophages in response to hyper-production of IL-6 (Ouchi et al., 2011).

Orthodontic treatment leads to change in the periodontium; foremost of these changes is the induction of inflammation. Measuring the levels of inflammation markers during OTM is therefore required. As MPO is an enzyme stored in granules of neutrophils, the level of MPO activity is directly linked to the number of PMNs in the tissue (Cao and Smith, 1989). In this study, the levels of MPO increased significantly after 1 hour (T2) in GCF of obese patients, and after 1 week (T3) in normal weight patients and continued until the end of alignment, with significantly higher levels in obese patients at all-time points (T1-T4). These results reflect the greater neutrophil activation in the periodontal tissues of obese patients, for whom obesity-related inflammation was exacerbated by orthodontic treatment. Previous studies that measured the levels of MPO in GCF of orthodontic patients reported that MPO levels were increased 2 hours after application, and then returned to the baseline level at the 7 and 14-day measurement points in both saliva and GCF (Marcaccini et al., 2010). This inconsistency could be related to different selection criteria, sample size, the assay used to analyse the samples and the effects of other additional variables. The high levels of MPO associated with obesity were also confirmed by recent studies reporting a significant increase in MPO levels in obese children compared to control groups (Marcovecchio et al., 2016, Varma et al., 2016).

The other inflammatory marker is CRP, produced by hepatocytes during inflammation. In this study, the levels of CRP were not changed in GCF during orthodontic alignment in both normal weight and obese patients. However, the levels steadily increased and became significantly higher in obese compared to normal weight patients after 1 week of appliance placement (T3) and at the end of orthodontic alignment (T4). These elevations can be explained by the effects of cytokines such as IL-1, IL-6 and TNF-α, produced as a response to orthodontic stimuli, which intensified with obesity (Khanna and Mali, 2010). These pro-inflammatory cytokines are produced at the sites of inflammation before being carried to the liver via systemic circulation, resulting ultimately in the increased secretion of acute inflammatory reactants such as CRP (Ide et al., 2004, Nibali et al., 2007).

The RANKL/RANK/OPG pathway relates to the bone metabolism; the concentrations of RANKL in GCF during OTM therefore need to be evaluated. The levels of RANKL were higher in obese patients than normal weight patients before and throughout the orthodontic alignment (T1-T4). This could be related to higher pro-inflammatory cytokines produced by adipocytes such as TNF-α, IL-1β and IL-6. During the alignment stage, the levels of RANKL were significantly increased at the end of alignment (T4) in both normal weight and obese groups, leading to higher osteoclastogensis and bone resorption at T4 – a reflection of the high bone remodelling in the postlag phase of OTM. As confirmed by previous study noticed a dramatic increase in osteoclastic activity during the transition from lag to postlag phase (Rody et al., 2001). Interestingly, this increase was significantly higher in obese patients, indicating that more bone resorption takes place with obesity, which explains the correlation between RANKL levels and the high rate of OTM in obese patients in our study. Increased RANKL levels also confirmed by previous studies that measured the levels of RANKL in GCF with OTM, demonstrating that RANKL levels were increased after 24 hours (Kawasaki et al., 2006) and after 42 days (Grant et al., 2013).

The MMPs play a key role in regulating various biological processes including PDL remodelling in both physiological and pathological conditions. The results of this study demonstrated a sharp increase in the levels of MMP9, 1 hour after appliance placement in both groups, with significantly higher increases in obese patients. This gradually reduced through the first week, but was still significantly higher than baseline levels at T3. It finally returned to baseline levels at T4. Such changes indicate that the levels of MMP9 were highly influenced by orthodontic force, which enhances the recruitment and activation of MMP9-producing cells, such as fibroblasts, monocytes and macrophages, in the first week of orthodontic force application. However, MMP8, which is mainly produced by neutrophils, demonstrated significantly higher levels in GCF of obese patients in comparison to normal weight patients, even before starting the orthodontic treatment and throughout the whole alignment stage. The only significant change between time points was observed after 1 hour of appliance placement (T2) in the normal weight group, after which it returned to baseline levels. This indicates that neutrophils are highly activated by obesity, leading to higher levels of MMP8 in obese patients. These changes were not significantly affected by the inflammatory conditions associated with orthodontic treatment.

The inhibitor TIMP1 reduces the damage done by MMPs to the ECM. In this study, the levels of TIMP1 were significantly higher in obese patients before and throughout the

entire alignment period. Between different time points, TIMP1 levels were significantly increased in GCF of normal weight patients from the first week (T3) forward. This could be an attempt to counter the increase in the levels of MMP9 and balance the ECM destruction by MMPs during remodelling. The findings of our study are in line with a previous study that measured MMPs and TIMPs in GCF with OTM and registered a significant increase in MMP9 and TIMPs 1 and 2 across all of the time points used in the study following force application (Grant et al., 2013). These changes also confirm the results of our cross-sectional study, in which MMP9 levels were significantly changed by orthodontic treatment, whereas MMP8 levels were significantly influenced by obesity.

The MMPs/TIMPs ratio is also important in assessing and monitoring the balance of ECM destruction and inhibition during OTM. Since the levels of TIMP1 were significantly higher in the obese group, this leads to reduced MMP9/TIMP1 and MMP8/TIMP1 ratios in obese group. As MMP9 showed more significant differences between time points of orthodontic alignment, more significant differences were also seen in the MMP9/TIMP1 ratio between time points and between normal and obese patients.

## 5.2.5 Conclusion

This prospective study investigated the levels of biomarkers in UWMS, GCF and serum before and during the orthodontic alignment stage in a cohort of obese and normal weight adolescent patients, undergoing fixed-appliance orthodontic treatment concluded that:

1- Salivary MPO levels reflect GCF MPO during the orthodontic alignment stage;
2- Bone remodelling marker RANKL levels were increased at the end of the alignment in GCF and serum;
3- Changes in inflammation, tissue and bone remodelling biomarkers during orthodontic alignment were mainly seen in GCF; and
4- The levels of tissue remodelling biomarkers, inhibitors, bone remodelling biomarkers, inflammation, and pro-inflammatory obesity biomarkers were higher in obese patients than in normal weight patients.

From the above findings, the null hypotheses were rejected, as obesity intensified some biochemical changes that take place during orthodontic alignment and GCF is more likely to express the biochemical changes associated with OTM.

# 5.3 The effect of obesity on orofacial pain following fixed-appliance placement

## 5.3.1 Introduction

Obesity in children and young adults has also been associated with poor mental health and psychosocial problems, including depression, anxiety and low self-esteem (Dreber et al., 2015, French et al., 1995). Collectively, these psychological factors can contribute to low quality-of-life indices for obese subjects (Buttitta et al., 2014, Heo et al., 2003), which can be improved with weight loss (Kaukua et al., 2003). There is an established relationship between obesity and chronic musculoskeletal pain, which can also manifest from childhood (Smith et al., 2014); however, there are also suggestions that obesity can influence peripheral pain perception and be a contributory factor for increased pain thresholds (Torensma et al., 2016, Price et al., 2013, Zahorska-Markiewicz et al., 1988, Zahorska-Markiewicz et al., 1983).

Orofacial pain is a common adverse effect of orthodontic treatment with fixed-appliances (Scheurer et al., 1996, Johal et al., 2014), particularly during the first few days after orthodontic appliance placement (Jones and Chan, 1992, Scott et al., 2008b, Woodhouse et al., 2015b, Scheurer et al., 1996, Johal et al., 2014, Otasevic et al., 2006, Rahman et al., 2015, Pringle et al., 2009). Archwire progression during treatment often produces further episodes of pain, which can collectively affect eating and sleeping, and impact on day-to-day activities (Brown and Moerenhout, 1991, Johal et al., 2014).

The experience of discomfort was registered in about 95% of orthodontic patients (Kvam et al., 1987, Scheurer et al., 1996), ranged from separator placement and band fitting (Ngan et al., 1989, Bergius et al., 2002, Bondemark et al., 2004), archwire placement and activations (Ngan et al., 1989, Scheurer et al., 1996, Firestone et al., 1999, Erdinç and Dinçer, 2004, Jones, 1984, Polat et al., 2005) that create tension and compression sites in the PDL space resulting in a painful sensation for the patients. Many factors can affect the pain sensation such as age (Ngan et al., 1989, Brown and Moerenhout, 1991, Jones and Chan, 1992, Scheurer et al., 1996, Fernandes et al., 1998, Jones and Richmond, 1985, Scott et al., 2008b), gender (Erdinç and Dinçer, 2004, Krishnan, 2007, Ngan et al., 1989, Scott et al., 2008b), cultural differences (Bergius et al., 2000, Krishnan, 2007), psychological

wellbeing (Brown and Moerenhout, 1991, Sergl et al., 1998), the emotional status and stress (Andreasen et al., 1997) and previous pain experience (Okeson, 2005).

Orthodontic force magnitude was considered the most effective factor, the lighter force, the more efficient and less traumatic during OTM (Reitan, 1956). Therefore, pain intensity may be directly correlated with the severity of crowding and the amount of the applied force.

The literature has widely documented that the pain caused by orthodontic appliances can be a primary factor affecting patient compliance (Brown and Moerenhout, 1991, Sergl et al., 1998, O'Connor, 2000), and a discontinuation of treatment or its early termination was reported mainly due to pain, functional, and aesthetic impairment caused by the appliances (Patel, 1989).

A number of studies have investigated the effect of different fixed-appliance systems on orthodontic pain, including bracket design (Papageorgiou et al., 2014a, Papageorgiou et al., 2016, Scott et al., 2008b, Bertl et al., 2012, Fleming et al., 2009, Rahman et al., 2015, Riley and Bearn, 2009, Pringle et al., 2009) and archwire composition, but there is little evidence to suggest that any particular appliance combination is associated with less pain than any other (Papageorgiou et al., 2014a, Riley and Bearn, 2009). However, orthodontic pain during the first week following the placement of fixed-appliances has been shown to have a consistent and reproducible pain profile, increasing during the first 24-72 hours and then reducing to baseline levels after a week (Woodhouse et al., 2015b, Fleming et al., 2009, Pringle et al., 2009), which provides a useful experimental model to investigate orofacial pain experience in different subjects.

#### 5.3.1.1 Aims and objectives

The main aim of this study was to investigate the effect of obesity on orthodontic pain in subjects undertaking routine orthodontic treatment with fixed appliances. Specifically, this prospective cohort study compared orthodontic pain experience during the first week of fixed-appliance therapy in two groups of adolescents aged between 12-18 years and classified as normal weight and obese based upon BMI.

To address this aim the following steps were undertaken:

1. Measure the maximum pain intensity using a self-reported 100 millimetre (mm) visual-analogue scale (VAS), immediately after (Ta) and at 4, 24, 72-hours and 1-week (Tb-Te, respectively) following appliance placement.

2. Measure the secondary outcomes included mean pain intensities, frequency and amount of oral analgesia taken during the observation period.

The null hypothesis was that there is no difference in the pain intensity during the initial phase of orthodontic alignment between normal weight and obese patients.

## 5.3.2 Methods

### 5.3.2.1 Participants

The 55 patients (28 normal weight and 27 obese) recruited for the prospective study were provided with a pain diary to complete over the week following placement of the fixed appliance. The diary recorded orthodontic pain immediately after appliance placement (Ta), at 4 hours (Tb), 24 hours (Tc), 72 hours (Td) and 1 week (Te) following the appointment, by means of a 100-mm VAS using the terms "Very comfortable" and "Very uncomfortable" as peripheral weightings (Seymour, 1982). The VAS score is the distance from the left end of the line to the point of the patients' mark, measured to the nearest mm. Each VAS score was measured on two separate occasions by the same operator (HFS); with the mean of the two measurements taken as the representative value. In addition to the VAS score, patients noted the consumption of oral analgesics that will later be expressed as logistic data in percentage and dosage that will be measured as amount of oral analgesics taken in milligram (mg). Each patient was free to take non-prescription oral analgesia as required. The pain diary was completed by the patient and returned at the following appointment (Figure 5.21).

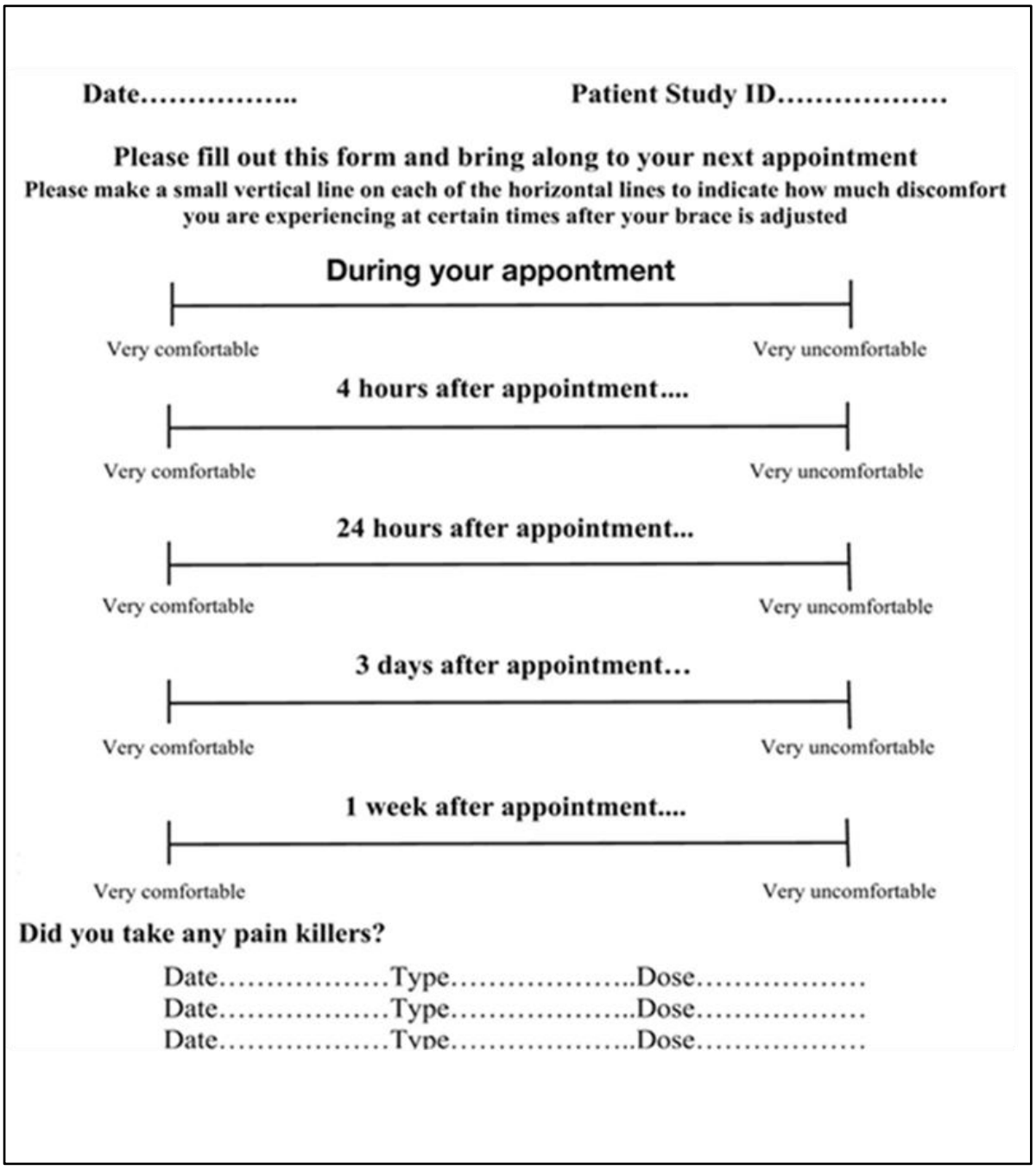



**Figure 5.21** Pain score diary supplied to the patients at fixed appliance placement appointment.

Pain diaries were coded appropriately so that both outcome assessor (HFS) and statistician were blinded to subject classification. The coding of data was broken after the end of the analysis and no breach of blinding was identified. A previous study showed through the intra-class correlation coefficient that the repeatability of measurements for VAS scores is almost perfect (Woodhouse et al., 2015b).

### 5.3.2.2 Study size

Study size calculation for this investigation was based upon the outcome of initial rate of orthodontic tooth alignment, which gave a required sample of 23 per group and has been described previously (Scott et al., 2008a). A post hoc power analysis was performed to assess, if this study had sufficient sample to identify a set 30% difference in maximum pain in obese compared to normal weight patients. Baseline maximum pain (74.63 mm) and common standard deviation (SD) for both groups (21.95 mm) originated from a previous study in the same clinical setting (Woodhouse et al., 2015b), while alpha for the independent t-test was set at 5%. The results of the power analysis indicated that the present study has 96% power to identify the specified effect on the primary outcome of maximum-pain experience.

### 5.3.2.3 Statistical analysis

Descriptive statistics were calculated to characterize the experimental groups, including means and standard deviations for continuous variables and frequencies for binary outcomes, after checking for normal distribution. Initial crude differences in baseline and outcome data were calculated with independent t-tests and chi-square tests.

The effect of obesity on the primary outcome (maximum-pain experienced during alignment) and all secondary outcomes was investigated using univariable (crude) and multivariable generalized estimation equation regression models with robust standard errors, adjusted for the confounding effect of baseline data (sex, age, baseline-irregularity, baseline-pain, plaque and gingival indices) and co-interventions (previous tooth extraction and use of analgesia during alignment).

Results are reported as unstandardized coefficients or Odds Ratios (ORs) for continuous and binary outcomes (the consumption of oral analgesics), respectively. In the analysis of mean pain across time-points, the model accounted for within-patient and time-point correlations.

Analysis of residuals was conducted to confirm no violation of the linear regression assumptions. All analyses were carried out using Stata 12.0 (Statacorp, College Station, TX, USA). A 2-tailed p-value of 0.05 was considered statistically significant with a 95% confidence interval (CI) for all tests.

## 5.3.3 Results

### 5.3.3.1 Participants

This prospective cohort study included 55 participants (27 males and 28 females) with a mean age of 15.1 (SD, 1.7) years and mean irregularity index of 7.6 (SD 2.4; 95% CI 6.9-8.2) mm. No patient drop-outs existed during the study period and all distributed pain diaries were collected and analysed. Mean overall BMI centile of the cohort was 24.7 (SD, 6.2) $kg/m^2$. Table 5.12 shows the baseline demographics and clinical characteristics of the two cohorts at start of treatment. The normal weight group had a mean BMI of 19.4 (SD, 2.2) whilst the obese group had a mean BMI of 30.2 (SD, 3.5) $kg/m^2$. Apart from BMI, there were no significant differences among groups for baseline characteristics.

**Table 5.12** Baseline demographics of subjects included in the prospective cohort study.

| | **Overall** | **Normal weight** | **Obese** | **P value** |
|---|---|---|---|---|
| Patients | 55 | 28 | 27 | |
| Male / female - n | 27/28 | 15/13 | 12/15 | 0.498* |
| Age - mean (SD) | 15.1 (1.7) | 15.1 (1.6) | 15.1 (1.9) | 0.991# |
| Plaque Index (SD) | 0.56 (0.32) | 0.57 (0.32) | 0.54 (0.31) | 0.745 |
| Gingival Index (SD) | 0.74 (0.39) | 0.74 (0.40) | 0.73 (0.38) | 0.934 |
| Crowding - mean (SD) | 7.6 (2.4) | 7.0 (2.3) | 8.2 (2.4) | 0.061# |
| BMI - mean (SD) | 24.7 (6.2) | 19.4 (2.2) | 30.2 (3.5) | <0.001# |
| Tooth extraction – n (%) | 8 (15%) | 4 (14%) | 4 (15%) | 0.956* |

SD, standard deviation; BMI, body mass index; * from chi-square test; # from independent t-test.

### 5.3.3.2 Primary outcome (maximum pain intensity during alignment)

Maximum-pain intensity across all time-points for the total sample was 73.7 mm (SD 14.8; 95% CI 69.8-77.7) with no significant differences among groups (Figure 5.22; Table 5.13; p=0.247). Moreover, at every single time point, the maximum-pain intensity was higher for the obese group compared to normal weight with a statistically significant difference at Td (p=0.034) (Table 5.13). A greater percentage of the obese group reported taking oral analgesia when compared to normal weight (74 versus 57 per cent), but this difference was not significant (p=0.187).

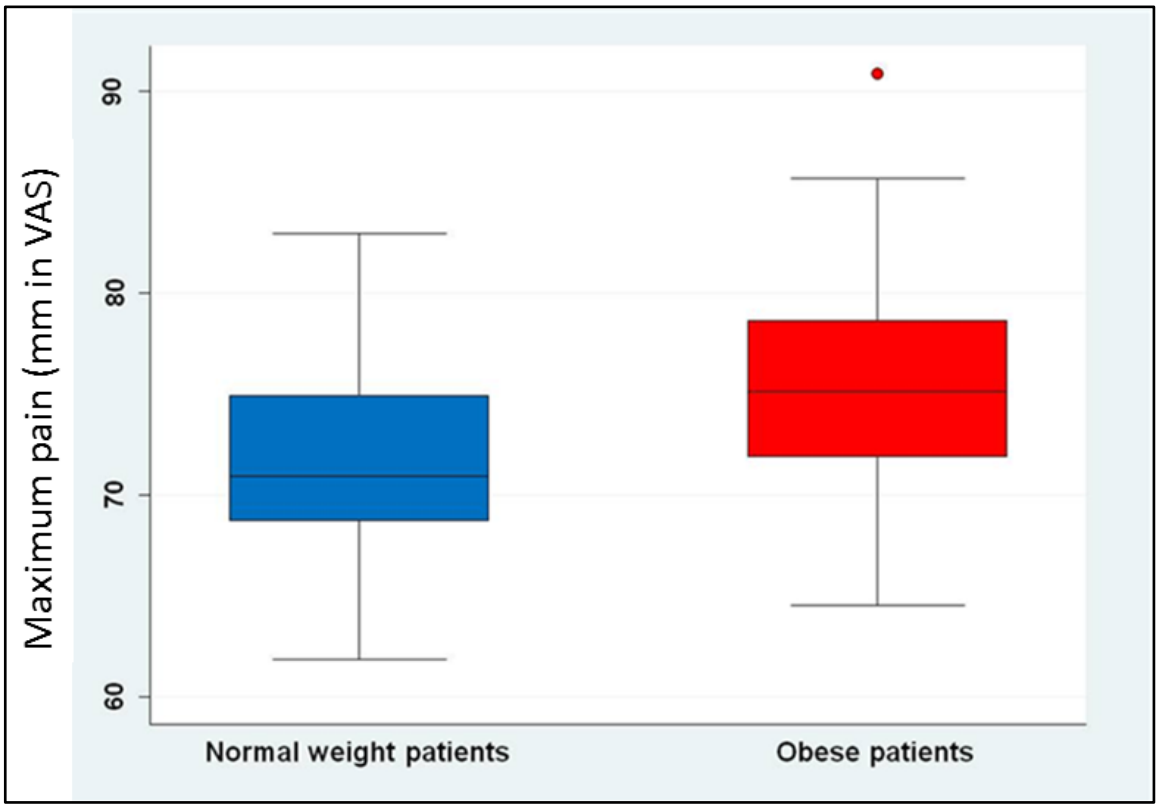



**Figure 5.22** Box plot showing the maximum pain during the whole alignment period (mm in VAS) from the multivariable regression analysis.

**Table 5.13** Results of patient-reported maximum pain and use of oral analgesia.

| Outcome | Overall | Normal weight | Obese | P value |
|---|---|---|---|---|
| Maximum pain in mm – mean (SD) | 73.7 (14.8) | 71.5 (15.9) | 76.1 (13.4) | 0.247* |
| VAS in mm Ta – mean (SD) | 18.5 (22.5) | 19.4 (23.8) | 17.6 (21.6) | 0.765* |
| VAS in mm Tb – mean (SD) | 47.3 (22.3) | 46.2 (22.6) | 48.4 (22.4) | 0.715* |
| VAS in mm Tc – mean (SD) | 70.3 (18.0) | 66.9 (20.2) | 74.0 (15.0) | 0.146* |
| VAS in mm Td – mean (SD) | 46.1 (19.4) | 40.6 (19.5) | 51.7 (18.0) | 0.034* |
| VAS in mm Te – mean (SD) | 12.5 (14.0) | 10.9 (13.5) | 14.2 (14.6) | 0.388* |
| Reported use of oral analgesia – n (%) | 36 (65) | 16 (57) | 20 (74) | 0.187# |
| Overall oral analgesia used in mg – mean (SD) | 1027.3 (1094.7) | 732.1 (937.7) | 1333.3 (1176.7) | 0.041* |

SD, standard deviation; VAS, visual analogue scale; *from independent t-test; #from chi square test.

Multivariable regression analysis indicated that there were no significant differences between normal weight and obese groups in terms of maximum reported pain during initial tooth alignment for a number of factors, including subject age, gender, irregularity, baseline reported pain, reported use of oral analgesia, and baseline plaque index (Table 5.14). However, there were significant differences between groups in terms of previous history of extractions (p=0.045) and baseline gingival index (p=0.010). Patients taking oral analgesics also reported slightly higher maximum pain compared to those who did not (represented by a difference of 5.98 mm in VAS scores), but this was not statistically significant (p=0.163).

**Table 5.14** Regression analyses on the outcome of maximum reported pain during the alignment period (Ta-Te) in mm in VAS scale.

| | **Univariable** | | | **Multivariable** | | |
|---|---|---|---|---|---|---|
| **Factor** | **Coefficient** | **95% CI** | **P value** | **Coefficient** | **95% CI** | **P value** |
| Obesity group (Ref: normal weight) | 4.65 | -3.03, 12.32 | 0.236 | 2.64 | -4.73, 10.03 | 0.482 |
| Age (per year increase) | | | | 1.26 | -0.54, 3.07 | 0.170 |
| Gender (Ref: female) | | | | 2.07 | -4.67, 8.81 | 0.547 |
| Irregularity at start | | | | 0.99 | -0.51, 2.48 | 0.195 |
| Tooth extraction (Ref: non-extraction) | | | | -8.29 | -16.39, -0.19 | 0.045 |
| Baseline pain (per VAS mm increase) | | | | 0.10 | -0.03, 0.24 | 0.130 |
| Use of oral analgesia (Ref: no) | | | | 5.98 | -2.43, 14.39 | 0.163 |
| Plaque index at Ta | | | | -11.74 | -24.40, 0.92 | 0.069 |
| Gingival index at Ta | | | | 13.17 | 3.21, 23.13 | 0.010 |

VAS, visual analogues scale; CI, confidence interval; Ref, reference; Ta, immediately after appliance placement.

#### 5.3.3.3 Secondary outcomes (mean pain at each time point and consumption of analgesics)

Figure 5.23 illustrates the mean-pain intensity of normal weight and obese groups at all time-points. Crude multivariable linear regression for mean pain at each time point indicated that the only significant predictors for pain intensity were time (Ta-Te) and reported baseline-pain (Figure 5.24; Table 5.15). However, after adjusting for confounders, obesity was associated with slightly higher (+4.42 mm in the VAS) mean pain at each time-point, which was statistically significant (p=0.017). Additionally, the interaction between obesity and time was not statistically significant (p=0.416), which indicates that although obese patients reported slightly higher pain than normal-weight patients at all time-points, the pain variation across time-points per se was similar in both groups.

Analysis of the consumption of oral analgesics (as a binary outcome) revealed no statistically significant association in either the univariable or the multivariable (adjusted) analyses (Table 5.16). Analysis of the total amount of analgesics consumed (as a continuous outcome) revealed statistically significant associations with obesity for both univariable and multivariable (adjusted) analyses (+601.20 mg, p=0.035 and +646.38 mg, p=0.023, respectively). There was also a significant association with age in the multivariable analysis, with older patients taking smaller amounts of analgesics (p=0.018). Also after accounting for confounders, obese patients were associated with higher overall amount of consumed oral analgesics (difference=646.38 mg) (Table 5.17).

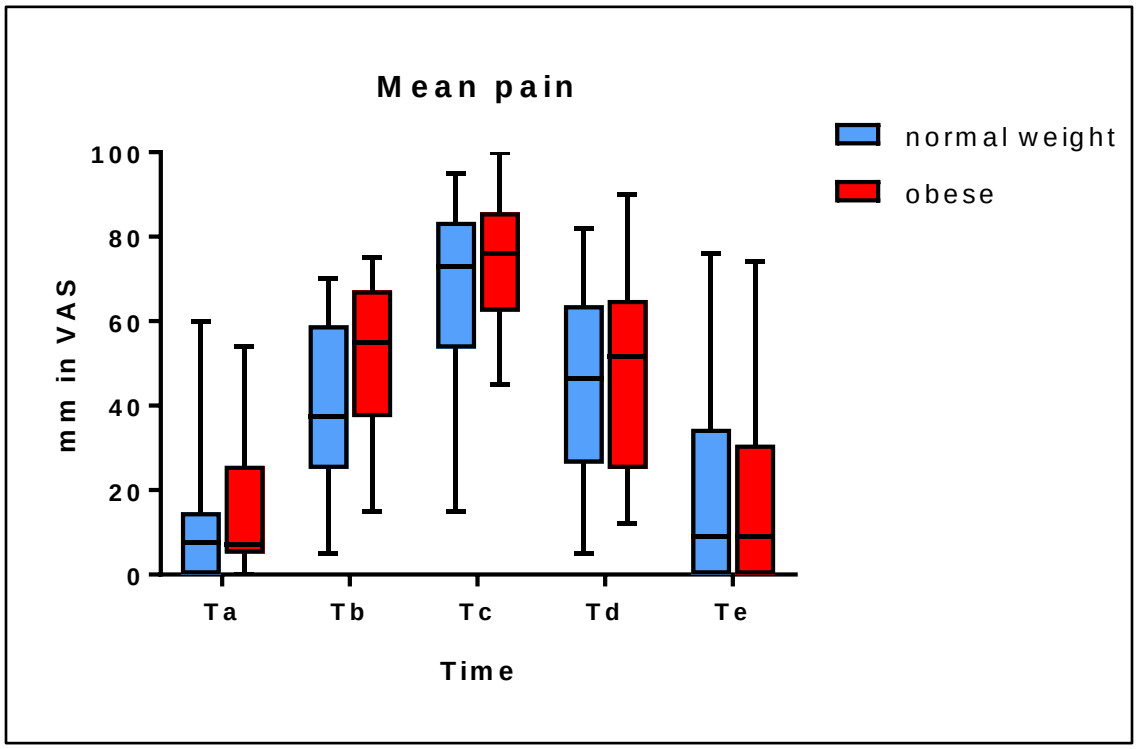



**Figure 5.23** Box plots showing the mean pain at each time-point Ta-Te (min to max). Ta, immediately after appliance placement; Tb, 4 hours; Tc, 24 hours; Td, 72 hours; and Te, 1 week following the placement of fixed orthodontic appliance appointment, vertical whiskers indicate minimum to maximum values.

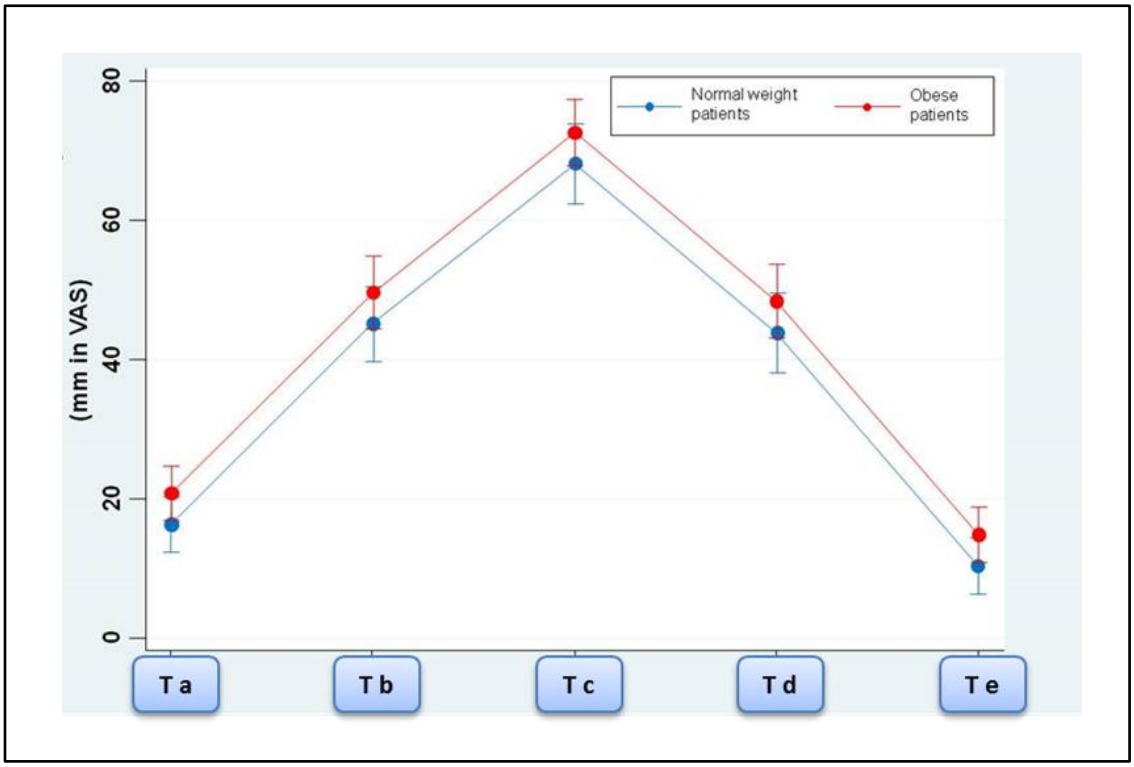



**Figure 5.24** Graph showing the mean pain at each time-point Ta-Te (mm in VAS) from the multivariable regression analysis. Ta, immediately after appliance placement; Tb, 4 hours; Tc, 24 hours; Td, 72 hours; and Te, 1 week following the placement of fixed orthodontic appliance appointment.

**Table 5.15** Regression analyses on the outcome of reported pain at each time-point in mm in VAS scale.

| | **Multivariable model 1** | | | **Multivariable model 2** | | |
|---|---|---|---|---|---|---|
| **Factor** | **Coefficient** | **95% CI** | **P value** | **Coefficient** | **95% CI** | **P value** |
| Obesity group (Ref: normal weight) | 3.52 | -3.05, 10.09 | 0.293 | 4.42 | 0.79, 8.05 | 0.017 |
| Time point | | | | | | |
| Ta | Ref | | | | | |
| Tb | 28.82 | 23.17, 34.47 | <0.001 | 28.81 | 23.17, 34.47 | <0.001 |
| Tc | 51.85 | 44.71, 59.00 | <0.001 | 51.85 | 44.71, 59.00 | <0.001 |
| Td | 27.58 | 20.22, 34.94 | <0.001 | 27.58 | 20.22, 34.94 | <0.001 |
| Te | -5.95 | -11.60, -0.29 | 0.039 | -5.95 | -11.60, -0.29 | 0.039 |
| Age (per year increase) | | | | 0.77 | -0.25, 1.78 | 0.137 |
| Gender (Ref: male) | | | | 1.96 | -1.78, 5.70 | 0.304 |
| Crowding (per mm increase) | | | | -0.46 | -1.26, 0.35 | 0.268 |
| Tooth extraction (Ref: non-extraction) | | | | -2.62 | -6.58, 1.34 | 0.194 |
| Baseline pain (per VAS mm increase) | | | | 0.43 | 0.34, 0.52 | <0.001 |
| Use of oral analgesia (Ref: no) | | | | 4.34 | 0.02, 8.67 | 0.049 |
| Plaque index at Ta | | | | -5.36 | -13.35, 2.63 | 0.189 |
| Gingival index at Ta | | | | 2.69 | -2.82, 8.20 | 0.339 |

*Interaction of obesity with time found to be non-significant (p=0.416) and was dropped from the model.
CI, confidence interval; Ref, reference; VAS, visual analogues scale; Ta, immediately after appliance placement; Tb, 4 hours; Tc, 24 hours; Td, 72 hours; and Te, 1 week following the placement of fixed orthodontic appliance appointment.

**Table 5.16** Regression analyses on the outcome of use of pain medication during the alignment phase (Ta-Te).

| | Univariable | | | Multivariable | | |
|---|---|---|---|---|---|---|
| **Factor** | **OR** | **95% CI** | **P value** | **OR** | **95% CI** | **P value** |
| Obesity group (Ref: normal weight) | 2.14 | 0.68, 6.78 | 0.194 | 1.93 | 0.55, 6.81 | 0.304 |
| Age (per year increase) | | | | 0.77 | 0.54, 1.11 | 0.164 |
| Gender (Ref: female) | | | | 0.81 | 0.25, 2.69 | 0.732 |
| Crowding (per mm increase) | | | | 1.10 | 0.84, 1.44 | 0.489 |
| Tooth extraction (Ref: non-extraction) | | | | 4.62 | 0.55, 38.69 | 0.159 |
| Baseline pain (per VAS mm increase) | | | | 1.01 | 0.99, 1.04 | 0.374 |
| Plaque index at Ta | | | | 1.29 | 0.10, 16.46 | 0.843 |
| Gingival index at Ta | | | | 0.95 | 0.08, 10.96 | 0.967 |

OR, odds ratio; CI, confidence interval; Ref, reference; VAS, visual analogues scale; T0, immediately after appliance placement.

**Table 5.17** Regression analyses on the outcome of overall dose of oral analgesia taken during the alignment phase (Ta-Te) in mg.

| | Univariable | | | Multivariable | | |
|---|---|---|---|---|---|---|
| **Factor** | **Coefficient** | **95% CI** | **P value** | **Coefficient** | **95% CI** | **P value** |
| Obesity group (Ref: normal weight) | 601.20 | -42.89, 1159.49 | 0.035 | 646.38 | 88.49, 1204.26 | 0.023 |
| Age (per year increase) | | | | -165.84 | -303.21, -28.47 | 0.018 |
| Gender (Ref: female) | | | | 80.36 | -428.89, 589.21 | 0.757 |
| Crowding (per mm increase) | | | | -3.50 | -117.02, 110.01 | 0.952 |
| Tooth extraction (Ref: non-extraction) | | | | 735.29 | -11.41, 1481.99 | 0.054 |
| Baseline pain (per VAS mm increase) | | | | 10.11 | -1.93, 22.14 | 0.100 |

Co, Coefficient; CI, confidence interval; Ref, reference; VAS, visual analogue scale.

## 5.3.4 Discussion

The commonality of obesity in Western society means that the implications of this condition are likely to affect increasing areas of modern healthcare and therefore, it is important that clinicians from all specialties understand potential issues associated with the care of obese patients.

During orthodontic treatment the perception of pain was correlated with various substances released as a result of change in blood flow caused by orthodontic force, such as substance P, histamine, enkephalin, dopamine, serotonin, glycine, glutamate, gamma-aminobutyric acid, PGs, leukotrienes, and cytokines (Davidovitch et al., 1988, Alhashimi et al., 2001, Yamasaki et al., 1984).

In the present study, we have investigated orthodontic pain during the first week following the placement of fixed orthodontic appliances in normal-weight and obese adolescents. We found that maximum reported pain across the first week was not significantly different between the two groups. However, mean pain was higher in the obese group when compared to normal-weight group at all individual time-points. This general trend for higher pain at each time-point and the fact that obesity did not have a modifying effect on the progression of pain through the study period (interaction $p>0.05$) suggests that obese patients reported generally higher pain levels than normal weight patients. Other psychological issues including depression, low self-esteem and anxiety are also correlated with pain experience during orthodontic treatment (Bergius et al., 2008, Ireland et al., 2017); however, these parameters have not been tested in this study. In addition, the higher amount of total analgesia taken by obese group may have no clinical importance. The effectiveness of analgesia is more likely related to the type of analgesic and mode of action (Bradley et al., 2007). For instance, acetaminophen inhibits the third isoform of cyclooxygenase enzyme ($COX_3$) that does not or slightly inhibits the formation of prostaglandins, whilst ibuprofen, piroxicam, aspirin and naproxen sodium inhibit $COX_1$ and $COX_2$ which control pain sensation through blocking the production of prostaglandins (Dionne and Berthold, 2001). Also it should be noted that the pain is a subjective sensation which can be influenced by cultural background, previous traumatic experience, sex and age (Brown and Moerenhout, 1991, Scheurer et al., 1996, Patel et al., 2011).

We found a pattern of orofacial pain intensity experienced during the week following placement of fixed-appliances that was similar to that seen in previous

investigations (Woodhouse et al., 2015b, Scott et al., 2008b, Pringle et al., 2009). This reinforces the consistency of pain response that is seen amongst individuals following placement of fixed orthodontic appliances and makes this a useful experimental model to investigate orofacial pain (Scott et al., 2008b, Woodhouse et al., 2015b, Fleming et al., 2009, Pringle et al., 2009). The prospective design (Papageorgiou et al., 2015b) and comparability of the experimental groups at baseline can also be considered strengths of the present study. Moreover, obesity was defined according to widely-accepted and reliable measures, which makes misclassification unlikely. Additionally, as the patients in the obese and normal-weight groups were similar apart from their BMI, no drop-outs were seen as we kept reminding the participants by phone, and even if the patient did not show up after 1 week, the diary was collected at the next appointment. The VAS measurement blinding was implemented, the respective risk for selection, attrition, and detection bias is low. Although we did not investigate orthodontic pain in the longer-term, there is evidence to indicate that there is consistency in the pattern during subsequent archwire changes (Woodhouse et al., 2015b, Scott et al., 2008b, Pringle et al., 2009). Although the VAS was explained clearly to the patients, there are some potential limitations, including the fact that the results depend mainly on the participants' reports, with the possibility of confusion between pain and discomfort, in addition to the accurate dose of analgesic components included in the taken tablets. In addition, it is worth mentioning that there is another statistical method for analysing the medical research data collected serially on subjects, which takes the form of a two stage method that uses summary measures (Matthews et al., 1990).

The relationship between obesity and oral disease is complex, but there are associations with reduced tooth number and irregular dental visits (Ostberg et al., 2012), periodontal disease (Suvan et al., 2011, Chaffee and Weston, 2010) dental caries status (Kantovitz et al., 2006) and increased risk of dental erosion (Tong et al., 2014). Moreover, the presence of oral disease seems to have a greater negative impact on quality-of-life in the morbidly obese when compared to normal weight subjects (Yamashita et al., 2015). Although the implications of obesity for orthodontic therapy are less clear, a number of potential associated factors have been discussed that may impact on treatment, which include psychosocial well-being, altered craniofacial growth, overall tissue turnover, and bone metabolism (Neeley and Gonzales, 2007, Mack et al., 2013, Giuca et al., 2012, Sadeghianrizi et al., 2005). In this study a number of associated factors were investigated such as sex, age, baseline-irregularity, baseline-pain, previous tooth extraction and use of

analgesia during alignment which may affect the pain sensation of individuals; also plaque and gingival inflammation, may induce some biochemical changes such as, IL-1β which is one of the inflammatory chemical mediators induce the secretion of pain-producing substances that have an impact on pain perception (Davidovitch et al., 1989).

The results of this investigation suggest that there are differences in the pain response between obese and normal-weight subjects during orthodontic treatment over the short-term with corresponding differences in the consumption of analgesics. This is in agreement with previous studies, which report that obesity can influence peripheral pain perception and be a contributory factor for increased pain thresholds (Price et al., 2013, Torensma et al., 2016, Zahorska-Markiewicz et al., 1983, Zahorska-Markiewicz et al., 1988). Possible explanations for this relationship include amongst others, peripheral factors such as skin thickness and innervation density (Shir et al., 2001), a possible nociceptive modulation through leptin (Kutlu et al., 2003) or interaction of ghrelin with central opioid mechanisms, which seems to have an anti-nociceptive effect on endogenous opioids (Guneli et al., 2010). In any case, prolonged orthodontic pain has been shown to have potential negative consequences on compliance (Sergl et al., 2000) and treatment completion (Sergl et al., 1998, Doll et al., 2000). As oral analgesics are effective in reducing orthodontic pain (Sandhu et al., 2016) it would seem prudent for clinicians to ensure that obese patients are provided with the necessary information and measures to ensure appropriate pain relief following the placement of fixed-appliances.

This is the first prospective study to investigate orthodontic pain in association with variation in body weight and provides evidence that informs clinical practice, both in orthodontics and other areas of medicine and dentistry. The results of this study are widely generalizable and applicable to the majority of obese and normal-weight adolescents aged between 12-18 years.

## 5.3.5 Conclusion

Based on the results of this prospective cohort study on pain experience during alignment in obese and normal-weight children with moderate to severe mandibular incisor irregularity, the following can be concluded:

1. The maximum-pain experienced during alignment was comparable in both normal weight and obese patients;

2. Obese patients experience statistically higher mean pain than normal weight patients at each time point during the first week after appliance placement; and
3. This was accompanied by a corresponding statistically significant higher consumption of pain medication.

# Chapter 6 General discussion

## 6.1 General discussion

The term obesity refers to an excess accumulation of body fat produced by adipocyte cells. Together, these cells form layers of adipose tissue, an endocrine organ responsible for the dysregulation of immune responses through the secretion of bioactive molecules called adipocytokines (Ouchi et al., 2011). Obesity increases the risk of chronic health problems and leads to serious comorbid conditions, clustered behavioural risk factors and, ultimately, high mortality rates. Recent meta-analyses have demonstrated a positive association between obesity and periodontitis (Suresh et al., 2016, Suvan et al., 2011, Keller et al., 2015), which appears to be more severe and more common in obese individuals than in normal weight individuals (Suvan et al., 2011). Since OTM mainly depends on periodontal tissues and alveolar bone remodelling, obesity can potentially affect orthodontic therapy, particularly the inflammatory mediators secreted in the periodontal sulcus.

Previous evidence concerning the relationship between obesity and orthodontics was limited to the implications of obesity for orthodontic diagnosis and treatment (Neeley and Gonzales, 2007) with recent evidence suggesting that increased BMI can be a risk factor for reduced cooperation, longer treatment duration and increased health-related problems during fixed appliance treatment (von Bremen et al., 2015). Currently no data exists in relation to OTM in obese patients. The main aim of this study was to examine the influence of obesity on OTM, from both clinical and biochemical perspectives, by studying the biochemical analysis of biomarkers for inflammation, tissue and bone remodelling and obesity, in different bio-fluids (UWMS, GCF and serum), alongside measurement of the rate of tooth movement and pain experience in both normal weight and obese subjects. For this purpose, the following hypotheses were tested:

- First hypothesis: Obesity does not change the levels of selected biomarkers.
  This was disproved and the null hypothesis was rejected as there were significant changes in the profile of several biomarkers in some bio-fluids, such as adiponectin, leptin, MMP8, TIMP1, RANKL, MPO and CRP, with obesity.
- Second hypothesis: Obesity does not change the levels of selected biomarkers in patients undergoing fixed appliance orthodontic treatment.

This was proved and the null hypothesis was accepted because although obesity affects markers in bio-fluids, orthodontic treatment drives even bigger effects.

- Third hypothesis: There is no difference between UWMS, GCF and serum in the biochemical analysis with OTM.

  This was disproved and the null hypothesis was rejected because changes in inflammation, tissue and bone remodelling biomarkers during orthodontic alignment were mainly seen in GCF.

- Fourth hypothesis: There is no difference in the orthodontic tooth alignment between normal weight and obese patients.

  This was disproved and the null hypothesis was rejected because obese patients showed a significantly higher rate of tooth movement compared with normal weight patients, although there was no significant reduction in the time required for the orthodontic alignment of obese patients.

- Fifth hypothesis: There is no difference in the pain intensity felt during the initial phase of orthodontic alignment between normal weight and obese patients.

  This was disproved and the null hypothesis was rejected because obese patients experienced higher mean pain than normal weight patients in the first week after appliance placement, accompanied by a concomitant consumption of analgesics that was also higher.

### 6.1.1 Rate of OTM

The measurement time points in this study were: T1 (before treatment); T2 (1 hour after appliance placement); T3 (1 week after); and T4 (end of orthodontic alignment). These points were selected in an attempt to follow the different biological changes underlying tooth movement. Based on human studies, it has been widely accepted that OTM follows a specific pattern in time with three phases (King et al., 1991, Rody et al., 2001): an initial phase, 1 - 2 days after force application, representing either rapid displacement of the tooth in the PDL space (Burstone, 1962) or bending of the alveolar bone (Baumrind, 1969); a lag phase, lasting 4 - 20 days, with relatively low or no displacement as a result of hyalinization of the PDL in areas of compression; and an acceleration postlag phase during which the rate of OTM reassumes after the removal of hyalinised tissue formed during the second phase (Burstone, 1962). Each phase is determined by specific reactions involving the recruitment of bone-resorbing and bone-forming precursors, as well as the extravasation

and chemotaxis of inflammatory cells (Krishnan and Davidovitch, 2006b). The activity of these cells can be measured biochemically by certain markers of inflammation and of tissue and bone metabolism.

In this study, significant association was found between the rate of tooth movement and the amount of initial tooth irregularity. This could be due to the fact that the same wire applies more force on teeth that are more crowded, resulting in greater displacement during the initial phase of tooth movement. This rate of tooth movement was significantly higher in obese patients after 1 week of archwire placement and at the end of alignment, largely due to the bone texture of obese subjects.

Existing information about the effect of body mass on bone texture varies. Some authors describe a positive effect of body mass on bone mineral density, as an adaptation to the increased load carried on the bone (Leonard et al., 2004, Clark et al., 2006); however, if this were also true for the bones surrounding the teeth, it would mean a slower OTM in obese patients. Other authors found that the change in hormones caused by obesity negatively affects bone quality, which could imply easier OTM (Janicka et al., 2007, Rhie et al., 2010, Viljakainen et al., 2011). This is verified by animal studies that reported a decrease in bone mineral density, bone mineral content and total skeleton area in rats fed on a high fat diet compared with those given a standard diet (Lac et al., 2008).

Rates of tooth movement were significantly higher in obese patients compared to normal weight patients during the first week (T1-T3; $p<0.001$), then decreased until the two groups showed comparable movement ($p=0.119$) between T3 (1 week) and T4 (the end of alignment). At the end of alignment, the obese group had displayed a significantly higher rate of overall tooth movement (T1-T4; $p=0.05$). One possible explanation is that the bone texture of obese subjects allows greater tooth displacement in the supporting bone, resulting in significantly faster tooth movement in the first week. In contrast, the bone remodelling process was less efficient in obese patients due to higher levels of leptin and pro-inflammatory cytokines that modulate bone metabolism (Krysiak et al., 2012, Carbone et al., 2012). These higher levels may lead to slower bone turnover, resulting in decelerated tooth movement. Taken together, these two parallel processes – one causing greater OTM and the other less efficient OTM – may explain why the orthodontic alignment period for obese patients remains comparable with that of normal weight patients.

It is worth noting that obesity and bone metabolism are interrelated, as both bone-forming cells (osteoblasts) and adipocytes are derived from a common multi-potential

mesenchymal stem cell (Greqoir et al., 1998); agents inhibiting adipogenesis therefore stimulate osteoblast differentiation (Gimble et al., 1996, David et al., 2007b, Sen et al., 2008) and vice versa (Beresford et al., 1992). Obesity affects bone metabolism through the upregulation of pro-inflammatory cytokines, such as IL-6 and TNF-α, that may increase bone resorption by stimulating osteoclast activity through the regulation of the RANKL/RANK/OPG pathway (Pfeilschifter et al., 2002). Furthermore, it provokes an increase in adipocyte-derived leptin (Canavan et al., 2005, Van Dielen et al., 2001) and a decrease in adiponectin, which stimulate macrophage transportation, accumulation and adhesion to endothelial cells (Maeda et al., 2002, Sierra-Honigmann et al., 1998). Additionally, the excess fatty acids found in a high-fat diet form unabsorbable, insoluble calcium soaps that interfere with proper calcium absorption in the intestine (Nelson et al., 1998, Carnielli et al., 1996, Lucas et al., 1997).

### 6.1.2 Biochemical analysis

Most human studies concerning the biology of OTM have focused on the analysis of different mediators involved in periodontal tissue and alveolar bone remodelling. Knowledge of biomarkers during OTM has clinical value when applied to considering aspects of orthodontic treatment such as the use of mechanical force to improve and shorten treatment time with minimal side effects (Kumar et al., 2015).

The next stage of investigation concerns how these biomarkers are produced. During orthodontic treatment, the applied forces affect the ECM of the PDL, resulting in cellular alteration, cytoskeletal configuration and temporary bioelectric signals that change the membrane polarity and ion channel activities. Such events lead to the production of neuropeptides, released from afferent nerve endings, as well as the vasodilatation of blood vessels in the PDL space, which causes the migration of leucocytes into the gingival sulcus. This migration creates a wide range of inflammatory mediators. The overall result of this interaction is the production of tissue-degrading enzymes and acids that induce cellular proliferation and differentiation, and promote wound healing and tissue remodelling. All of these biochemical changes take place across all stages (earlier and later) of active orthodontic treatment and even during the retention period following orthodontic therapy (Hill and Orth, 1998, Davidovitch, 1995, Sandy et al., 1993). The status of the tissue around the teeth during OTM can be examined by analysing the GCF in the surrounding

sulcus to assess and modify the orthodontic treatment procedure. A major limitation of GCF studies is the small sample volume for analysis (typically less than 0.5 µl) that is obtained from healthy sites (Canavarro et al., 2013). The small volume of GCF limits the number of analytes that can be conveniently studied by traditional ELISA; however, the introduction of the multianalyte microsphere assay allows the simultaneous quantification of several targets in a single assay. The multiplex, magnetic bead-based Luminex assay was used in this study to detect multiple biomarkers in UWMS, GCF and serum.

Data from the study showed that there was no correlation between biomarker levels and plaque and gingival scores. Changes of biomarkers can therefore be attributed to inflammation taking place during OTM, rather than bacterial plaque or gingivitis.

Obesity biomarkers (adiponectin, leptin and resistin) showed that the levels of adiponectin and leptin, which are mainly produced by adipocytes, were higher in serum than in GCF and were lowest in UWMS, thus indicating their systemic secretion from adipocytes directly into circulation. In contrast, the highest levels of resistin were found in GCF, followed by serum and finally UWMS. This is due to the fact that, although it is an obesity biomarker, it is mainly produced by neutrophils, macrophages with very little presence in adipocytes in humans (Patel et al., 2003, Rosen and Spiegelman, 2006). These findings are in agreement with previous studies that reported higher levels of resistin in GCF than serum (Patel and Raju, 2014).

In this study, the levels of adiponectin were lower, whereas the levels of leptin and resistin were higher in obese subjects compared with normal weight subjects. These results are in agreement with other studies that report a decrease in the levels of adiponectin (Ouchi et al., 1999, Joseph et al., 2002, Kadowaki and Yamauchi, 2005, Ashley et al., 2011) and an increase in the levels of leptin (Considine et al., 1996) and resistin (Piestrzeniewicz et al., 2008) with obesity; however, another study reported no association between resistin levels with BMI (Mamali et al., 2012), which confirms the findings of the cross-sectional study. The reason for this inconsistency is unclear, but it could be related to differences in patient age and stage of orthodontic treatment.

The prospective study highlighted that, during orthodontic alignment, the only significant change in adipokine levels was the reduction of adiponectin levels in serum at the end of the alignment (T4), with significantly greater reduction in the obese group than the normal weight group. While the overall levels of these adipokines during orthodontic alignment were comparable to those before treatment, they still showed higher leptin and

resistin, and lower adiponectin levels in obese patients. The remarkably higher levels of leptin and resistin in GCF of obese patients can explain the association between these proteins and the higher rate of tooth movement in obese patients.

The other group of biomarkers is the tissue remodelling biomarkers such as MMP8, MMP9 and their inhibitor TIMP1. These biomarkers have been chosen for this study because they are involved in collagen turnover and catalyse the initial degradation of the major ECM proteins in PDL remodelling during OTM, which can also be delayed or inhibited with TIMPs (Holmbeck et al., 2004).

Data from cross-sectional and prospective studies showed that MMP8 mostly changed with obesity, MMP9 mostly changed with OTM, and TIMP1 changed with both. MMP8, mainly produced by the PMNs (Mäntylä et al., 2003, Ingman et al., 2005), cleaves the interstitial collagens I, II, and III (Raffetto and Khalil, 2008). MMP9, mainly secreted by fibroblasts, monocyte and macrophages, (Bourboulia and Stetler-Stevenson, 2010) cleaves elastin and collagen IV, V and X (Tayebjee et al., 2004a, Tayebjee et al., 2004b). TIMPs inhibit MMPs by developing 1:1 enzyme-inhibitor complexes (Gomez et al., 1997).

In the gingival sulcus, where the local effects of orthodontic forces take place, GCF showed an increase in MMP9 levels within the first week of orthodontic treatment in both normal weight and obese groups, without marked differences between the two groups. On the contrary, MMP8 and TIMP1 levels were significantly higher in the obese group before and throughout the orthodontic alignment, with limited changes between time points during the orthodontic treatment. These findings confirm the results of GCF analysis in the cross-sectional study, in which only MMP9 (but not MMP8 or TIMP1) increased significantly in the GCF of obese patients with orthodontic appliances. These results could be due to the impact of MMP9 on the degradation of elastin and different types of collagen, such as IV, V, and X (Tayebjee et al., 2004a, Tayebjee et al., 2004b) that may be increased during orthodontic treatment and enhanced by the inflammatory conditions of obesity. Additionally, MMP9 plays a role, not only in the degradation of collagen matrices, but also in the migration of osteoclast precursors to sites of bone resorption (Ishibashi et al., 2006). This may explain the elevation in the levels of MMP9, but not MMP8, at 1 hour after the placement of the fixed orthodontic appliance and throughout the first week, affecting the MMP9/TIMP1 ratio.

Circulating MMP8, MMP9 and TIMP1 showed comparable levels in obese and normal weight patients in the prospective study; however, at the end of alignment (T4), the

levels of MMP8 and MMP8/TIMP1 ratio were significantly higher in obese patients. Furthermore, although circulating MMP8 levels were not significantly higher in obese patients with fixed orthodontic appliances in the cross-sectional study; the MMP9 levels were significantly increased. Such events may indicate the possible systemic effects of fixed appliance orthodontic treatment in obese patients and increase the risk of systemic diseases, such as cardiovascular diseases and metabolic syndromes associated with high levels of MMP8 in the blood (Turu et al., 2006, Souza-Costa et al., 2007, Koh et al., 2005).

The other biomarkers investigated in this study were the inflammation biomarkers including MPO and CRP. Levels of MPO increased with obesity and with orthodontic treatment, indicating a greater number of neutrophils in response to the inflammatory condition triggered by obesity or orthodontic therapy or both. The changes in the levels of MPO can be clearly seen in saliva and GCF, in agreement with the findings of previous studies (Marcaccini et al., 2010, Navarro-Palacios et al., 2014). The regression analysis demonstrated that the association between this enzyme and the rate of tooth movement can be explained by the high levels of MPO in GCF with obesity and significant changes in orthodontic treatment.

CRP, which acts as a diagnostic marker for inflammation, tissue damage and low-grade systemic inflammation (Ridker and Silvertown, 2008, Paraskevas et al., 2008), was elevated in the obese group and mainly detected in serum. During orthodontic treatment, the prospective study explored whether there were any significant changes in the levels of CRP at all-time points in the three tested bio-fluids. However, the significantly higher levels of circulating CRP in obese subjects, before and through the whole orthodontic alignment, was reflected in the GCF of those patients, resulting in significantly higher levels of CRP in GCF of obese patients compared to normal weight patients after 1 week of fixed orthodontic appliance placement. This may indicate that the orthodontic treatment of obese subjects has a systemic effect, as a result of the hyper-production of IL-1, IL-6 and TNF-α in the gingival sulcus, combined with an orthodontic force that stimulates the hepatocyte to produce more CRP in the serum (Ide et al., 2004, Nibali et al., 2007) that then diffuses to GCF.

The last biomarker measured in this study is the bone remodelling cytokine RANKL, the key to osteoclastogensis and bone resorption. Produced mainly by osteoblasts, it is highly influenced by pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. The

highest levels of RANKL were therefore found in GCF and were significantly greater in obese subjects (Halade et al., 2011, Xu et al., 2013).

Interestingly, the data of this study demonstrated that the levels of RANKL in GCF and serum were significantly increased at the end of alignment (T4), which reflects the postlag phase in which a higher rate of bone resorption take place. These findings are in agreement with a previous study (Grant et al., 2013). In relation to obesity, RANKL levels in GCF and serum were remarkably higher in obese than normal weight patients, both before and throughout the alignment stage. However, salivary RANKL showed a different pattern than GCF and serum, with reduced levels in obese subjects compared with the normal weight group. Although these differences were not significant enough during the first week, it appeared significant at the end of alignment (T4). This is supported by the significant reduction of salivary RANKL levels with obesity in the control cohort of our cross-sectional study. The reason behind such variation is possibly related to the effect of obesity on the diffusion of RANKL from serum and GCF to saliva, rather than the salivary glands themselves. This can be supported by previous studies, reporting that salivary RANKL originates from GCF rather than salivary glands (Sakellari et al., 2008, Buduneli et al., 2009, Tang et al., 2009).

### 6.1.3 The levels of biomarkers in different bio-fluids

Alongside advances in biology, interest in the potential use of oral biomarkers in periodontal diagnostics and prognostics has soared. Saliva has been proposed as the most useful sample to use for periodontal monitoring, due to the fact that it is unlike serum or GCF in terms of easy, rapid, safe and non-invasive collection; a cost-efficient method of disease screening, without the need for highly trained professionals. Simple processing and analysis is required; and salivary analytes are very stable, which makes it feasible for patients to produce the sample at home and post it to the laboratory. Investigating GCF analytes in relation to OTM is also an easy, non-invasive process. It is more reflective of the site-specific view, rather than the overall oral status of an individual supplied by saliva alone. This is due to the fact that the supracrestal gingival collagen fibres are not completely resorbed or degraded during OTM; rather, they are compressed or retracted during tooth dislocation (Redlich et al., 1996). This mechanism may be important in the maintenance of a healthy periodontium during orthodontic movement. The constant

presence of supracrestal collagen during tooth movement might restrict the diffusion of mediators from the PDL to the gingival margin. Serum analysis in obesity research is also required in this study, as adipocytes produce adipokines and cytokines directly into circulation.

The data of this study showed that some biomarkers are systemically produced, such as adiponectin, leptin and CRP, which are directly secreted from adipocytes and hepatic cells into the circulation. These had the highest levels in serum, followed by GCF, and the least in UWMS. Other biomarkers such as resistin, MMPs and their inhibitor TIMPs, MPO, and RANKL showed their highest levels in GCF, which may indicate local production, as well as their diffusion from blood. Biomarkers that are locally produced are able to play a role in the biological mechanisms that take place in the PDL under normal conditions, such as tooth eruption and physiological tooth movement. They also potentially constitute a response to mechanical stimuli, such as OTM, and inflammatory conditions such as periodontitis. Furthermore, in this study, it was noticed that obesity may affect the diffusion of some mediators from blood to saliva, as the different patterns of RANKL and leptin in saliva were observed in comparison to those in GCF and serum.

### 6.1.4 UWMS and GCF flow rate

Since the concentration of biomarkers is highly influenced by flow rate, the UWMS and GCF flow rate were measured in this study. From these results, it can be seen that UWMS flow rate was not affected by obesity, as a comparable UWMS flow rate was observed in normal weight and obese adults aged 18 to 45 without orthodontic treatment (control cohort in cross-sectional study), and at baseline (T1) before the placement of orthodontic appliances for patients aged 12 to 18 (in the prospective study). These findings are in agreement with previous studies measuring the salivary flow rate in obese adults (FenolI-Palomares et al., 2004) and children (Pannunzio et al., 2010). However, UWMS flow rate was influenced by OTM, regardless of BMI, and significantly increased in both normal weight and obese patients after 1 hour of fixed orthodontic appliances placement, as seen at T2-T4 in the prospective study. Such modifications can be considered as a physiological response to the mechanical stimulation of fixed orthodontic appliance components such as bands, tubes, brackets and wires that can influence the UWMS flow rate. This was previously reported by other researchers (Li et al., 2009) and might affect the salivary

constituents (Edgar, 1992). This explanation is based on the phenomenon that the placement of a foreign body in the mouth stimulates saliva secretion and increases the flow rate (Forsberg et al., 1992); that the salivary parameters become stable after 6 months of fixed orthodontic appliance placement can be seen as a form of adaptation (Li et al., 2009), which can explain a comparable UWMS flow rate in normal weight and obese adults aged 18 to 45 with orthodontic treatment (orthodontic treatment cohort in cross-sectional study).

Conversely, the GCF flow rate increased with both obesity and with orthodontic treatment, as was clearly seen in the prospective study in which GCF flow rate had a higher starting point in obese patients than in normal weight patients before the treatment. This then significantly increased in both groups 1 hour after fixed orthodontic appliance placement, with remarkably greater levels in the obese group until the end of the orthodontic alignment (T1-T4). The cross-sectional study also showed increased GCF flow rate with obesity in the control cohort, without orthodontic treatment, while the cohort undergoing orthodontic treatment showed comparable GCF flow rates in obese and normal weight patients. Such diversity could be related to the age of patients and the stage of the orthodontic treatment, as the prospective study was started before, and continued during, the orthodontic alignment of patients aged 12 to 18, while the cross-sectional study was undertaken at the final alignment stage of fixed appliance orthodontic treatment of patients aged 18 to 45. These explanations are based on the findings of previous studies highlight that the GCF flow rate increased with inflammation (Goodson, 2003), showed greater quantity in adolescents than adults regardless of the presence of any stimuli (Ren et al., 2002) and its constitutes were affected by local and systemic conditions (Ebersole, 2003). Interestingly, the increase in GCF flow rate with orthodontic treatment could be attributed to the effect of orthodontic force on blood vessels, rather than biochemical changes in the ECM (Baldwin et al., 1999). The data of this study confirms previous studies (Basaran et al., 2006, Karacay et al., 2007); however, other investigations reported that the mechanical stress of orthodontic appliance has no impact on GCF flow rate during OTM (Uematsu et al., 1996b, Miyajima et al., 1990).

### 6.1.5 Pain during orthodontic treatment

The pain experienced during orthodontic treatment is an inflammatory pain that results from vascular changes, recruitment and differentiation of inflammatory and immune cells,

and the production of neurogenic and inflammatory mediators in response to the mechanical stimuli by the orthodontic appliance. It has been reported that OTM and pain are two dependent and interrelated biological events, incorporating local inflammation and the production of various mediators that act on sensory nerve endings to incite painful sensations (Sacerdote and Levrini, 2012, Nimeri et al., 2013). Therefore, the mechanisms underlying orthodontic pain lie in the interaction of vascular, cellular and chemical components of periodontal inflammatory responses, induced by orthodontic forces. Pain signals pass through the periodontal sensory nerve endings to the pain perception areas of the brain's sensory cortex (Long et al., 2016).

In relation to obesity, there is evidence that the increased production of inflammatory mediators, by the macrophage accumulated in adipose tissues, plays a role in pain modulation (Wellen and Hotamisligil, 2003). The mechanism behind this could be related to the elevated levels of pro-inflammatory mediators, TNF-α, IL-6 and CRP, and decreased anti-inflammatory mediators like adiponectin in blood, leading to systemic inflammation (Blüher et al., 2005). This consequently affects the peripheral and central pain transmission system and results in hyperalgesia (Bastard et al., 2006).

In relation to biochemical analysis, studies have focused on the correlation between GCF biochemical mediators and pain intensity during orthodontic treatment. The intensity of pain 1 hour after the separator placement was associated with prostaglandin E2 levels, and after 24 hours, it was associated with IL-1β levels (Giannopoulou et al., 2006). Others found the correlation of pain intensity with prostaglandin E2 levels extends to 12, 24 and 72 hours after the application of labial orthodontic force (Yao et al., 2003). Substance P was also recognized as a mediator that initiates dental pain and inflammation, which remarkably increased in patients undergoing orthodontic treatment (Sacerdote and Levrini, 2012).

From the above, it has been postulated that increased levels of cytokines influence and contribute to the sensation of pain by increasing the sensitization of nociceptors (Srikanth Babu et al., 2012, Stürmer et al., 2005). This can explain the higher mean of pain in the obese group at all-time points, which in turn leads to a higher quantity of oral analgesic intake by obese subjects. Pain-related cytokines were not included in the biomarkers selected in this study, but the outcome was clearly similar to the findings of previous investigations of pain with orthodontic treatment (Scott et al., 2008b, Woodhouse et al., 2015b, Pringle et al., 2009).

## 6.2 Conclusion

The results of this thesis suggest there are clinical and biochemical implications of obesity for orthodontic treatment.

### 6.2.1 The clinical implications

During orthodontic alignment, the rate of OTM was higher in obese patients, particularly in the first week, due to the hyper-production of pro-inflammatory biomarkers that modify bone texture with obesity. This leads to initial faster displacement of teeth in the surrounding bone, and then slows down; ending with an insignificant difference in the time required for the orthodontic alignment compared with normal weight patients.

Obese patients experience higher mean pain than normal weight patients, accompanied by higher consumption of analgesics; therefore, obese patients need to be provided with the necessary information and measures to ensure appropriate pain relief following the placement of fixed-appliances.

### 6.2.2 The biochemical implications

GCF analysis is more likely to express the biochemical changes during OTM in both normal weight and obese subjects, although some changes were expressed in serum and saliva. Thus, the biochemical analysis of GCF can be used clinically to monitor and predict the outcome of orthodontic treatment. For instance, the optimum force can be determined to achieve the most rapid tooth movement with minimal side effects, optimize the required retention period, and minimize the chance of relapse when these changes have disappeared.

The adipokines leptin and resistin, the inflammatory-marker myeloperoxidase, and the bone remodelling cytokine RANKL, were significantly different in the GCF of obese and normal weight patients. As these biomarkers are associated with observed rates of tooth movement, further investigation of these biomarkers is recommended with OTM.

Sequentially, both obesity and orthodontic force influence the levels of some biomarkers in some bio-fluids. At the early stage of fixed appliance orthodontic treatment, the effect of obesity was greater on the level of these biomarkers; however, by the advanced stage, the orthodontic treatment itself had bigger effects.

Obesity, to some extent, can reduce the levels of salivary leptin and RANKL by affecting their diffusion from serum and GCF, which showed higher levels of leptin and RANKL in obese subjects.

Salivary MPO levels can reflect the levels of MPO in GCF of normal weight and obese subjects both with and without orthodontic treatment; therefore, the recommendation is to measure MPO non-invasively in UWMS.

Salivary adiponectin has a small contribution from serum, but there also appears to be a contaminating protein that reacts with several antibodies in Western blots and ELISA, which is unlikely to be adiponectin.

## 6.3 Future work

The outcomes of this thesis suggest the need for further clinical and biochemical investigations.

Clinically, the data of this thesis highlights potential implications for orthodontic treatment in obese subjects during the alignment stage of fixed appliance orthodontic therapy. Additional avenues of study also exist to compare the stability of teeth following the orthodontic treatment in obese and normal weight patients.

Additionally, the thesis showed that the rate of tooth movement was comparable in normal weight and obese patients between the end of the first week and the end of alignment (T3-T4). Therefore, it would be interesting to also measure the rate of tooth movement at every follow up visit, to assess closely the difference in the speed of tooth movement between the two groups.

Biochemically, from the data presented in this thesis, the bone remodelling cytokine RANKL a key for osteoclastogensis, was significantly different between obese and normal weight patients and is associated with OTM. Previous literature reported that the RANKL/OPG balancing system is crucial in the regulation of osteoclastogenesis (Trouvin and Goeb, 2010, Bae et al., 2011). It would be valuable to measure the inhibitor OPG and RANKL/OPG ratio with obesity. This was not possible in this study, because OPG could not be included in the same base panel of the Luminex kit.

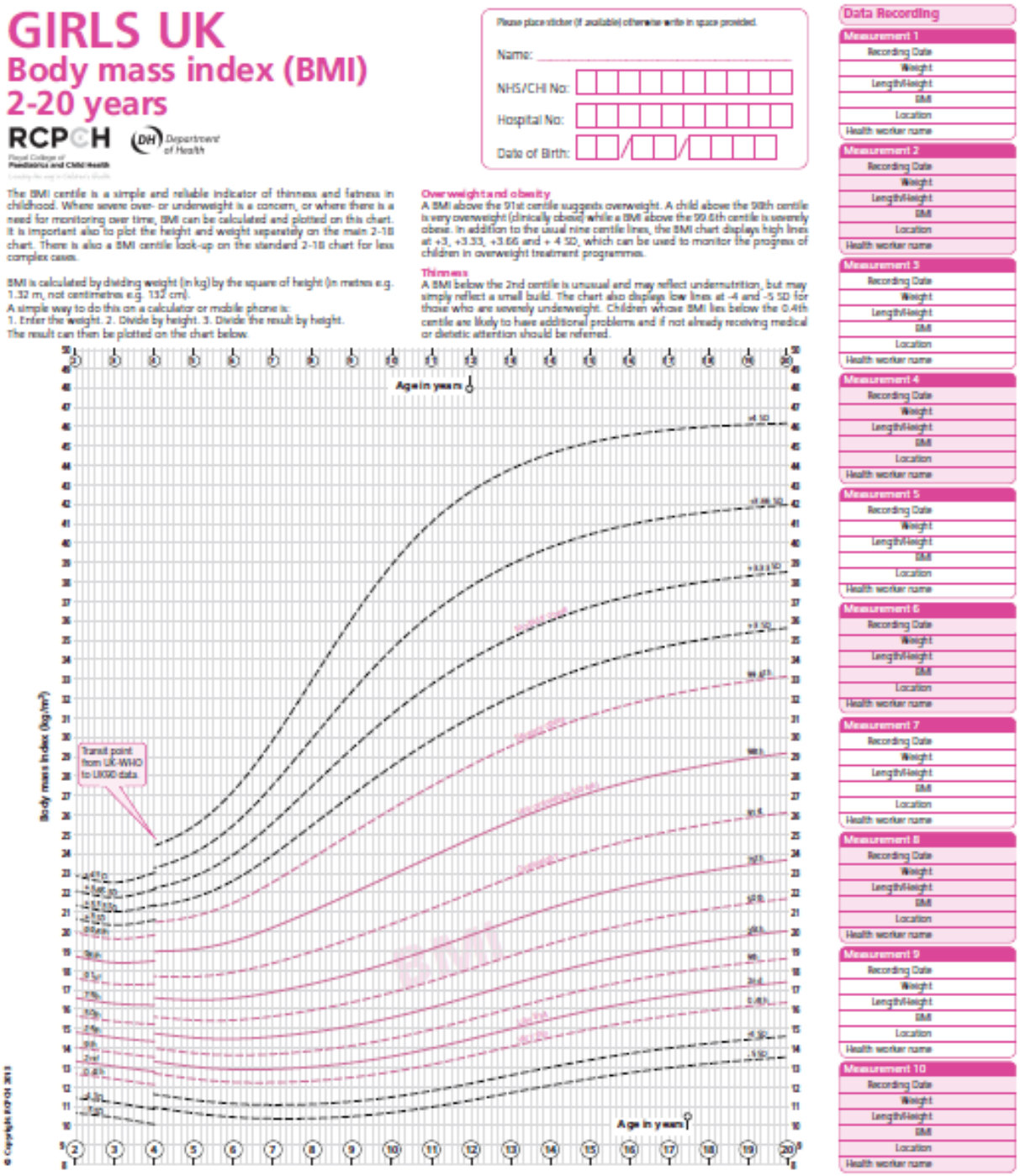
The other finding of this thesis and previous investigations (Junior et al., 2011, Almeida et al., 2015) was the significant changes in the levels of MMPs, starting within hours of orthodontic appliance placement, then shortly returning to baseline levels. It would

be interesting to investigate these enzymes multiple times within the first day and on a daily basis within the first week to monitor the fluctuating changes in their levels.

Finally, the data presented in this thesis provides evidence that salivary adiponectin has a small contribution from serum, but there also appears to be a contaminating protein that reacts with several antibodies in Western blots and ELISA, which is unlikely to be adiponectin. It also would be interesting to identify these proteins and their biological activities.

# Appendices

**Appendix 1** Girls UK Body Mass Index (BMI) 2-20 years (Royal College of Paediatric and Child Health (RCPCH), UK. (WHO, 2013).



GIRLS UK

Body mass index (BMI)

2-20 years

RCPCH

Department of Health

Please place sticker (if available) otherwise write in space provided.

Name:

NHS/CHI No:

Hospital No:

Date of Birth:

The BMI centile is a simple and reliable indicator of thinness and fatness in childhood. Where severe over- or underweight is a concern, or where there is a need for monitoring over time, BMI can be calculated and plotted on this chart. It is important also to plot the height and weight separately on the main 2-18 chart. There is also a BMI centile look-up on the standard 2-18 chart for less complex cases.

BMI is calculated by dividing weight (in kg) by the square of height (in metres e.g. 1.32 m, not centimetres e.g. 132 cm).
A simple way to do this on a calculator or mobile phone is:
1. Enter the weight. 2. Divide by height. 3. Divide the result by height.
The result can then be plotted on the chart below.

**Overweight and obesity**
A BMI above the 91st centile suggests overweight. A child above the 98th centile is very overweight (clinically obese) while a BMI above the 99.6th centile is severely obese. In addition to the usual nine centile lines, the BMI chart displays high lines at +3, +3.33, +3.66 and + 4 SD, which can be used to monitor the progress of children in overweight treatment programmes.

**Thinness**
A BMI below the 2nd centile is unusual and may reflect undernutrition, but may simply reflect a small build. The chart also displays low lines at -4 and -5 SD for those who are severely underweight. Children whose BMI lies below the 0.4th centile are likely to have additional problems and if not already receiving medical or dietetic attention should be referred.

Age in years

Body mass index (kg/m²)

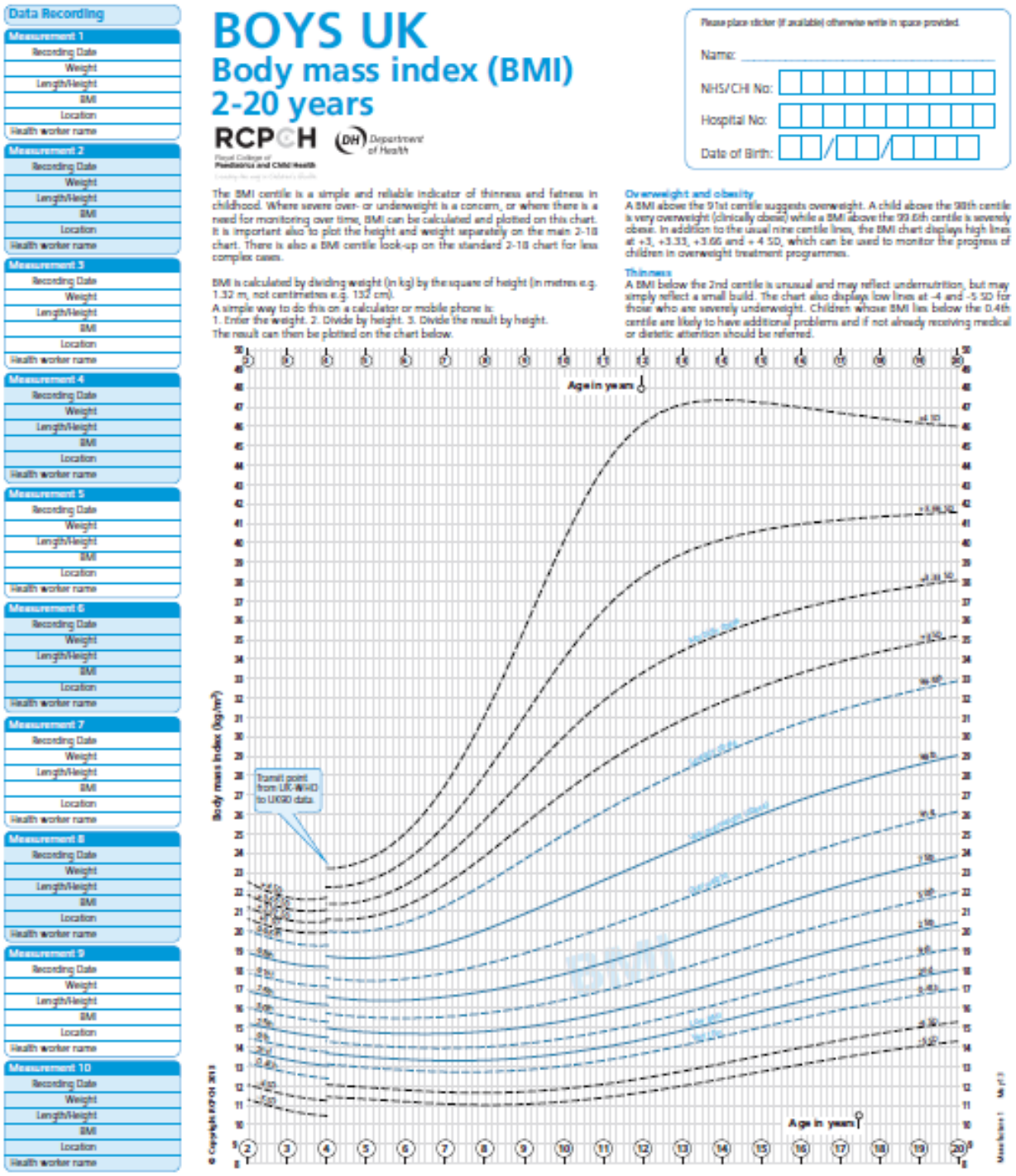
Transit point from UK-WHO to UK90 data

Data Recording

Measurement 1
Measurement 2
Measurement 3
Measurement 4
Measurement 5
Measurement 6
Measurement 7
Measurement 8
Measurement 9
Measurement 10

Recording Date
Weight
Length/Height
BMI
Location
Health worker name

**Appendix 2** Boys UK Body Mass Index (BMI) 2-20 years (Royal College of Paediatric and Child Health (RCPCH), UK. (WHO, 2013).



**Appendix 3** List of proteins detected by mass spectrometry in positive bands of serum sample

| | |
|---|---|
| 1 | Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 |
| 2 | Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 SV=2 |
| 3 | Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3 |
| 4 | Plasminogen OS=Homo sapiens GN=PLG PE=1 SV=2 |
| 5 | Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2 |
| 6 | Complement factor B OS=Homo sapiens GN=CFB PE=1 SV=2 |
| 7 | Carbonic anhydrase 1 OS=Homo sapiens GN=CA1 PE=1 SV=2 |
| 8 | Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1 |
| 9 | Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2 |
| 10 | Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2 |
| 11 | Complement C4-A OS=Homo sapiens GN=C4A PE=1 SV=1 |
| 12 | Inter-alpha-trypsin inhibitor heavy chain H2 OS=Homo sapiens GN=ITIH2 PE=1 SV=2 |
| 13 | Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1 |
| 14 | Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2 |
| 15 | Transitional endoplasmic reticulum ATPase OS=Homo sapiens GN=VCP PE=1 SV=4 |
| 16 | Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1 |
| 17 | Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 |
| 18 | Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3 |
| 19 | Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3 PE=1 SV=2 |
| 20 | Catalase OS=Homo sapiens GN=CAT PE=1 SV=3 |
| 21 | Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1 |
| 22 | Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2 |
| 23 | Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 |
| 24 | Protein AMBP OS=Homo sapiens GN=AMBP PE=1 SV=1 |
| 25 | Cathepsin D OS=Homo sapiens GN=CTSD PE=1 SV=1 |
| 26 | Apolipoprotein B-100 OS=Homo sapiens GN=APOB PE=1 SV=2 |
| 27 | Carbonic anhydrase 2 OS=Homo sapiens GN=CA2 PE=1 SV=2 |
| 28 | Inter-alpha-trypsin inhibitor heavy chain H4 OS=Homo sapiens GN=ITIH4 PE=1 SV=4 |
| 29 | Ubiquitin-60S ribosomal protein L40 OS=Homo sapiens GN=UBA52 PE=1 SV=2 |
| 30 | Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 |
| 31 | Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=1 SV=2 |
| 32 | Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3 |
| 33 | Caspase-14 OS=Homo sapiens GN=CASP14 PE=1 SV=2 |
| 34 | Complement component C7 OS=Homo sapiens GN=C7 PE=1 SV=2 |
| 35 | Calmodulin-like protein 5 OS=Homo sapiens GN=CALML5 PE=1 SV=2 |
| 36 | Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2 |
| 37 | Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1 |
| 38 | Prolactin-inducible protein OS=Homo sapiens GN=PIP PE=1 SV=1 |
| 39 | Apolipoprotein D OS=Homo sapiens GN=APOD PE=1 SV=1 |
| 40 | Ceruloplasmin OS=Homo sapiens GN=CP PE=1 SV=1 |
| 41 | Complement C2 OS=Homo sapiens GN=C2 PE=1 SV=2 |
| 42 | Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1 |
| 43 | Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=3 |
| 44 | Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2 |

Continue Appendix 3

| | |
|---|---|
| 45 | Protein S100-A7 OS=Homo sapiens GN=S100A7 PE=1 SV=4 |
| 46 | Complement component C6 OS=Homo sapiens GN=C6 PE=1 SV=3 |
| 47 | Inter-alpha-trypsin inhibitor heavy chain H1 OS=Homo sapiens GN=ITIH1 PE=1 SV=3 |
| 48 | Inter-alpha-trypsin inhibitor heavy chain H3 OS=Homo sapiens GN=ITIH3 PE=1 SV=2 |
| 49 | Prelamin-A/C OS=Homo sapiens GN=LMNA PE=1 SV=1 |
| 50 | Ubiquitin carboxyl-terminal hydrolase 5 OS=Homo sapiens GN=USP5 PE=1 SV=2 |
| 51 | Ig gamma-2 chain C region OS=Homo sapiens GN=IGHG2 PE=1 SV=2 |
| 52 | Complement C5 OS=Homo sapiens GN=C5 PE=1 SV=4 |
| 53 | Galectin-7 OS=Homo sapiens GN=LGALS7 PE=1 SV=2 |
| 54 | Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1 SV=1 |
| 55 | Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1 |
| 56 | Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4 |
| 57 | Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2 |
| 58 | Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2 |
| 59 | Cathepsin B OS=Homo sapiens GN=CTSB PE=1 SV=3 |
| 60 | Ezrin OS=Homo sapiens GN=EZR PE=1 SV=4 |
| 61 | Plectin OS=Homo sapiens GN=PLEC PE=1 SV=3 |
| 62 | Vitronectin OS=Homo sapiens GN=VTN PE=1 SV=1 |
| 63 | Ig heavy chain V-III region TIL OS=Homo sapiens PE=1 SV=1 |
| 64 | Desmocollin-1 OS=Homo sapiens GN=DSC1 PE=1 SV=2 |
| 65 | Calmodulin-like protein 3 OS=Homo sapiens GN=CALML3 PE=1 SV=2 |
| 66 | Gamma-glutamylcyclotransferase OS=Homo sapiens GN=GGCT PE=1 SV=1 |
| 67 | Gasdermin-A OS=Homo sapiens GN=GSDMA PE=2 SV=4 |
| 68 | Plasma protease C1 inhibitor OS=Homo sapiens GN=SERPING1 PE=1 SV=2 |
| 69 | Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5 |
| 70 | Serpin B3 OS=Homo sapiens GN=SERPINB3 PE=1 SV=2 |
| 71 | Ig gamma-4 chain C region OS=Homo sapiens GN=IGHG4 PE=1 SV=1 |
| 72 | Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1 |
| 73 | Tropomyosin alpha-3 chain OS=Homo sapiens GN=TPM3 PE=1 SV=1 |
| 74 | 14-3-3 protein zeta/delta OS=Homo sapiens GN=YWHAZ PE=1 SV=1 |
| 75 | Vitamin D-binding protein OS=Homo sapiens GN=GC PE=1 SV=1 |
| 76 | Ubiquitin-like modifier-activating enzyme 1 OS=Homo sapiens GN=UBA1 PE=1 SV=3 |
| 77 | L-lactate dehydrogenase B chain OS=Homo sapiens GN=LDHB PE=1 SV=2 |
| 78 | 78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2 |
| 79 | Ganglioside GM2 activator OS=Homo sapiens GN=GM2A PE=1 SV=4 |
| 80 | Cathepsin L2 OS=Homo sapiens GN=CTSL2 PE=1 SV=2 |
| 81 | Histidine-rich glycoprotein OS=Homo sapiens GN=HRG PE=1 SV=1 |
| 82 | Vinculin OS=Homo sapiens GN=VCL PE=1 SV=4 |
| 83 | Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=3 |
| 84 | Filaggrin OS=Homo sapiens GN=FLG PE=1 SV=3 |
| 85 | Heat shock protein beta-1 OS=Homo sapiens GN=HSPB1 PE=1 SV=2 |
| 86 | Malate dehydrogenase, cytoplasmic OS=Homo sapiens GN=MDH1 PE=1 SV=4 |
| 87 | Adiponectin OS=Homo sapiens GN=ADIPOQ PE=1 SV=1 |
| 88 | Cartilage oligomeric matrix protein OS=Homo sapiens GN=COMP PE=1 SV=2 |

**Appendix 4** List of proteins detected by mass spectrometry in positive band of saliva sample

| | |
|---|---|
| 1 | Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 |
| 2 | Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 SV=2 |
| 3 | Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2 |
| 4 | Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1 |
| 5 | Myeloperoxidase OS=Homo sapiens GN=MPO PE=1 SV=1 |
| 6 | Protein disulfide-isomerase OS=Homo sapiens GN=P4HB PE=1 SV=3 |
| 7 | Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2 |
| 8 | Complement C4-A OS=Homo sapiens GN=C4A PE=1 SV=1 |
| 9 | Pyruvate kinase isozymes M1/M2 OS=Homo sapiens GN=PKM PE=1 SV=4 |
| 10 | Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1 |
| 11 | Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2 |
| 12 | Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1 |
| 13 | Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3 |
| 14 | Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3 PE=1 SV=2 |
| 15 | Moesin OS=Homo sapiens GN=MSN PE=1 SV=3 |
| 16 | Catalase OS=Homo sapiens GN=CAT PE=1 SV=3 |
| 17 | Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1 |
| 18 | Ig alpha-2 chain C region OS=Homo sapiens GN=IGHA2 PE=1 SV=3 |
| 19 | Coronin-1A OS=Homo sapiens GN=CORO1A PE=1 SV=4 |
| 20 | Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 |
| 21 | Cystatin-S OS=Homo sapiens GN=CST4 PE=1 SV=3 |
| 22 | Nucleobindin-2 OS=Homo sapiens GN=NUCB2 PE=1 SV=2 |
| 23 | Ubiquitin-60S ribosomal protein L40 OS=Homo sapiens GN=UBA52 PE=1 SV=2 |
| 24 | Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=1 SV=2 |
| 25 | Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 |
| 26 | Caspase-14 OS=Homo sapiens GN=CASP14 PE=1 SV=2 |
| 27 | Adenylyl cyclase-associated protein 1 OS=Homo sapiens GN=CAP1 PE=1 SV=5 |
| 28 | Brain acid soluble protein 1 OS=Homo sapiens GN=BASP1 PE=1 SV=2 |
| 29 | Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=6 |
| 30 | Prolactin-inducible protein OS=Homo sapiens GN=PIP PE=1 SV=1 |
| 31 | Calreticulin OS=Homo sapiens GN=CALR PE=1 SV=1 |
| 32 | SPARC-like protein 1 OS=Homo sapiens GN=SPARCL1 PE=1 SV=2 |
| 33 | DnaJ homolog subfamily C member 3 OS=Homo sapiens GN=DNAJC3 PE=1 SV=1 |
| 34 | Ig gamma-2 chain C region OS=Homo sapiens GN=IGHG2 PE=1 SV=2 |
| 35 | Annexin A11 OS=Homo sapiens GN=ANXA11 PE=1 SV=1 |
| 36 | Leukocyte elastase inhibitor OS=Homo sapiens GN=SERPINB1 PE=1 SV=1 |
| 37 | Polymeric immunoglobulin receptor OS=Homo sapiens GN=PIGR PE=1 SV=4 |
| 38 | Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1 |
| 39 | Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4 |
| 40 | Glucose-6-phosphate isomerase OS=Homo sapiens GN=GPI PE=1 SV=4 |
| 41 | Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isofo |
| 42 | Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2 |
| 43 | Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2 |
| 44 | Ezrin OS=Homo sapiens GN=EZR PE=1 SV=4 |

Continue Appendix 4

| | |
|---|---|
| 45 | Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1 |
| 46 | Basic salivary proline-rich protein 1 OS=Homo sapiens GN=PRB1 PE=1 SV=2 |
| 47 | Vitronectin OS=Homo sapiens GN=VTN PE=1 SV=1 |
| 48 | Ig heavy chain V-III region TIL OS=Homo sapiens PE=1 SV=1 |
| 49 | IgGFc-binding protein OS=Homo sapiens GN=FCGBP PE=1 SV=3 |
| 50 | Kallikrein-1 OS=Homo sapiens GN=KLK1 PE=1 SV=2 |
| 51 | Mucin-5B OS=Homo sapiens GN=MUC5B PE=1 SV=3 |
| 52 | Carbonic anhydrase 6 OS=Homo sapiens GN=CA6 PE=1 SV=3 |
| 53 | Plastin-2 OS=Homo sapiens GN=LCP1 PE=1 SV=6 |
| 54 | Ig gamma-4 chain C region OS=Homo sapiens GN=IGHG4 PE=1 SV=1 |
| 55 | Tropomyosin alpha-3 chain OS=Homo sapiens GN=TPM3 PE=1 SV=1 |
| 56 | BPI fold-containing family B member 1 OS=Homo sapiens GN=BPIFB1 PE=2 SV=1 |
| 57 | Cystatin-SN OS=Homo sapiens GN=CST1 PE=1 SV=3 |
| 58 | Glucose-6-phosphate 1-dehydrogenase OS=Homo sapiens GN=G6PD PE=1 SV=4 |
| 59 | Nicotinamide phosphoribosyltransferase OS=Homo sapiens GN=NAMPT PE=1 SV=1 |
| 60 | EH domain-containing protein 1 OS=Homo sapiens GN=EHD1 PE=1 SV=2 |
| 61 | Nucleobindin-1 OS=Homo sapiens GN=NUCB1 PE=1 SV=4 |
| 62 | Immunoglobulin J chain OS=Homo sapiens GN=IGJ PE=1 SV=4 |
| 63 | Protein disulfide-isomerase A3 OS=Homo sapiens GN=PDIA3 PE=1 SV=4 |
| 64 | Cytochrome b-245 heavy chain OS=Homo sapiens GN=CYBB PE=1 SV=2 |
| 65 | T-complex protein 1 subunit alpha OS=Homo sapiens GN=TCP1 PE=1 SV=1 |

# Bibliography

A CATALáN, M., NAKAMOTO, T. & E MELVIN, J. 2009. The salivary gland fluid secretion mechanism. *The Journal of Medical Investigation,* 56**,** 192-196.

ADAR, S. D., D'SOUZA, J., MENDELSOHN-VICTOR, K., JACOBS, D. R., CUSHMAN, M., SHEPPARD, L., THORNE, P. S., BURKE, G. L., DAVIGLUS, M. L. & SZPIRO, A. A. 2015. Markers of inflammation and coagulation after long-term exposure to coarse particulate matter: a cross-sectional analysis from the multi-ethnic study of atherosclerosis. *Environmental health perspectives,* 123**,** 541-548.

AHIMA, R. S. & FLIER, J. S. 2000. Leptin. *Annual review of physiology,* 62**,** 413-437.

AKALIN, F. A., BALTACIOĞLU, E., ALVER, A. & KARABULUT, E. 2007. Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. *Journal of clinical periodontology,* 34**,** 558-565.

AKUAILOU, E.-N., VIJAYAGOPAL, P., IMRHAN, V. & PRASAD, C. 2013. Measurement and validation of the nature of salivary adiponectin. *Acta diabetologica,* 50**,** 727-730.

AL-QAWASMI, R. A., HARTSFIELD, J. K., EVERETT, E. T., FLURY, L., LIU, L., FOROUD, T. M., MACRI, J. V. & ROBERTS, W. E. 2003. Genetic predisposition to external apical root resorption. *American journal of orthodontics and dentofacial orthopedics,* 123**,** 242-252.

AL-ZAHRANI, M. S., BISSADA, N. F. & BORAWSKI, E. A. 2003. Obesity and periodontal disease in young, middle-aged, and older adults. *Journal of Periodontology,* 74**,** 610-615.

ALHASHIMI, N., FRITHIOF, L., BRUDVIK, P. & BAKHIET, M. 2001. Orthodontic tooth movement and de novo synthesis of proinflammatory cytokines. *American Journal of Orthodontics and Dentofacial Orthopedics,* 119**,** 307-312.

ALIKHANI, M., RAPTIS, M., ZOLDAN, B., SANGSUWON, C., LEE, Y. B., ALYAMI, B., CORPODIAN, C., BARRERA, L. M., ALANSARI, S. & KHOO, E. 2013. Effect of micro-osteoperforations on the rate of tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 144**,** 639-648.

ALMEIDA, R. C., CAPELLI JR, J. & TELES, R. P. 2015. Levels of gingival crevicular fluid matrix metalloproteinases in periodontally compromised teeth under orthodontic forces. *The Angle Orthodontist,* 85**,** 1009-1014.

AMĂLINEI, C., CĂRUNTU, I.-D. & BĂLAN, R. A. 2007. Biology of metalloproteinases. *Romanian Journal of Morphology and Embryology* 48**,** 323-334.

AMERONGEN, A. & VEERMAN, E. 2002. Saliva–the defender of the oral cavity. *Oral diseases,* 8**,** 12-22.

ANASTASI, G., CORDASCO, G., MATARESE, G., RIZZO, G., NUCERA, R., MAZZA, M., MILITI, A., PORTELLI, M., CUTRONEO, G. & FAVALORO, A. 2008. An immunohistochemical, histological, and electron-microscopic study of the human periodontal ligament during orthodontic treatment. *International journal of molecular medicine,* 21**,** 545-554.

ANDRADE, I., TADDEI, S. R. & SOUZA, P. E. Inflammation and tooth movement: the role of cytokines, chemokines, and growth factors. Seminars in Orthodontics, 2012. Elsevier, 257-269.

ANDREASEN, J. O., PETERSON, J. & LASKIN, D. M. 1997. Textbook and color atlas of tooth impactions. *American Journal of Orthodontics and Dentofacial Orthopedics,* 112**,** 354.

APAJALAHTI, S., SORSA, T., RAILAVO, S. & INGMAN, T. 2003. The in vivo levels of matrix metalloproteinase-1 and-8 in gingival crevicular fluid during initial orthodontic tooth movement. *Journal of Dental Research,* 82**,** 1018-1022.

APS, J. K. & MARTENS, L. C. 2005. Review: the physiology of saliva and transfer of drugs into saliva. *Forensic Science International,* 150**,** 119-131.

ARIFFIN, S. H. Z., ELLIAS, M. F., WAHAB, R. M. A., BAKAR, Y. & SENAFI, S. 2010. Profiles of lactate dehydrogenase, tartrate resistant acid phosphatase and alkaline photophatase in saliva during orthodontic treatment. *Sains Malaysiana,* 39**,** 405-412.

ASHLEY, D. T., O'SULLIVAN, E. P., DAVENPORT, C., DEVLIN, N., CROWLEY, R. K., MCCAFFREY, N., MOYNA, N. M., SMITH, D. & O'GORMAN, D. J. 2011. Similar to adiponectin, serum levels of osteoprotegerin are associated with obesity in healthy subjects. *Metabolism,* 60**,** 994-1000.

ATUĞ ÖZCAN, S. S., CEYLAN, İ., ÖZCAN, E., KURT, N., DAĞSUYU, İ. M. & ÇANAKCI, C. F. 2014. Evaluation of oxidative stress biomarkers in patients with fixed orthodontic appliances. *Disease markers,* 2014.

AUNE, D., SEN, A., PRASAD, M., NORAT, T., JANSZKY, I., TONSTAD, S., ROMUNDSTAD, P. & VATTEN, L. J. 2016. BMI and all cause mortality: systematic review and non-linear dose-response meta-analysis of 230 cohort studies with 3.74 million deaths among 30.3 million participants. *BMJ,* 353**,** i2156.

AURER, A., JORGIĆ-SRDJAK, K., PLANČAK, D., STAVLJENIĆ-RUKAVINA, A. & AURER-KOŽELJ, J. 2005. Proinflammatory factors in saliva as possible markers for periodontal disease. *Collegium antropologicum,* 29**,** 435-439.

AVELLAN, N.-L., SORSA, T., TERVAHARTIALA, T., MäNTYLä, P., FORSTER, C. & KEMPPAINEN, P. 2005. Painful tooth stimulation elevates matrix metalloproteinase-8 levels locally in human gingival crevicular fluid. *Journal of dental research,* 84**,** 335-339.

BABAEI, Z., MOSLEMI, D., PARSIAN, H., KHAFRI, S., POURAMIR, M. & MOSAPOUR, A. 2015. Relationship of obesity with serum concentrations of leptin, CRP and IL-6 in breast cancer survivors. *Journal of the Egyptian National Cancer Institute,* 27**,** 223-229.

BAE, T. S., KIM, B. I., SOH, Y. J. & NEPAL, M. 2011. Ethyl Docosahexaenoate and Its Acidic Form Increase Bone Formation by Induction of Osteoblast Differentiation and Inhibition of Osteoclastogenesis. *Biomolecules & Therapeutics,* 19**,** 70-76.

BAHCECI, M., GOKALP, D., BAHCECI, S., TUZCU, A., ATMACA, S. & ARIKAN, S. 2007. The correlation between adiposity and adiponectin, tumor necrosis factor α, interleukin-6 and high sensitivity C-reactive protein levels. Is adipocyte size associated with inflammation in adults? *Journal of endocrinological investigation,* 30**,** 210-214.

BALDUCCI, L., RAMACHANDRAN, A., HAO, J., NARAYANAN, K., EVANS, C. & GEORGE, A. 2007. Biological markers for evaluation of root resorption. *Archives of oral biology,* 52**,** 203-208.

BALDUS, S., HEESCHEN, C., MEINERTZ, T., ZEIHER, A. M., EISERICH, J. P., MUNZEL, T., SIMOONS, M. L. & HAMM, C. W. 2003. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. *Circulation,* 108**,** 1440-1445.

BALDWIN, P., PENDER, N. & LAST, K. 1999. Effects on tooth movement of force delivery from nickel-titanium archwires. *The European Journal of Orthodontics,* 21**,** 481-489.

BARBIERI, G., SOLANO, P., ALARCóN, J. A., VERNAL, R., RIOS-LUGO, J., SANZ, M. & MARTíN, C. 2012. Biochemical markers of bone metabolism in gingival crevicular fluid during early orthodontic tooth movement. *The Angle Orthodontist,* 83**,** 63-69.

BARTZELA, T., TURP, J. C., MOTSCHALL, E. & MALTHA, J. C. 2009. Medication effects on the rate of orthodontic tooth movement: a systematic literature review. *American Journal of Orthodontics and Dentofacial Orthopedics,* 135**,** 16-26.

BASARAN, G., ÖZER, T., KAYA, F. A., KAPLAN, A. & HAMAMCI, O. 2006. Interleukine-1β and tumor necrosis factor-α levels in the human gingival sulcus during orthodontic treatment. *The Angle orthodontist,* 76**,** 830-836.

BASSUK, S. S., RIFAI, N. & RIDKER, P. M. 2004. High-sensitivity C-reactive protein: clinical importance. *Current problems in cardiology,* 29**,** 439-493.

BASTARD, J.-P., MAACHI, M., LAGATHU, C., KIM, M. J., CARON, M., VIDAL, H., CAPEAU, J. & FEVE, B. 2006. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *European cytokine network,* 17**,** 4-12.

BAUMRIND, S. 1969. A reconsideration of the propriety of the "pressure-tension" hypothesis. *American journal of orthodontics,* 55**,** 12-22.

BAUR, L. A. & O'CONNOR, J. 2004. Special considerations in childhood and adolescent obesity. *Clinics in Dermatology,* 22**,** 338-344.

BECERIK, S., ÖZTURK, V. Ö., ATMACA, H., ATILLA, G. & EMINGIL, G. 2012. Gingival crevicular fluid and plasma acute-phase cytokine levels in different periodontal diseases. *Journal of Periodontology,* 83**,** 1304-1313.

BECK, J., GARCIA, R., HEISS, G., VOKONAS, P. S. & OFFENBACHER, S. 1996. Periodontal disease and cardiovascular disease. *Journal of periodontology,* 67**,** 1123-1137.

BELO, V. A., SOUZA-COSTA, D. C., LANA, C. M., CAPUTO, F. L., MARCACCINI, A. M., GERLACH, R. F., BASTOS, M. G. & TANUS-SANTOS, J. E. 2009. Assessment of matrix metalloproteinase (MMP)-2, MMP-8, MMP-9, and their inhibitors, the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in obese children and adolescents. *Clinical biochemistry,* 42**,** 984-990.

BERESFORD, J., BENNETT, J., DEVLIN, C., LEBOY, P. & OWEN, M. 1992. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *Journal of cell science,* 102**,** 341-351.

BERG, A. H. & SCHERER, P. E. 2005. Adipose tissue, inflammation, and cardiovascular disease. *Circulation research,* 96**,** 939-949.

BERGIUS, M., BERGGREN, U. & KILIARIDIS, S. 2002. Experience of pain during an orthodontic procedure. *European journal of oral sciences,* 110**,** 92-98.

BERGIUS, M., BROBERG, A. G., HAKEBERG, M. & BERGGREN, U. 2008. Prediction of prolonged pain experiences during orthodontic treatment. *American Journal of Orthodontics and Dentofacial Orthopedics,* 133**,** 339. e1-339. e8.

BERGIUS, M., KILIARIDIS, S. & BERGGREN, U. 2000. Pain in orthodontics. *Journal of Orofacial Orthopedics/Fortschritte der Kieferorthopädie,* 61**,** 125-137.

BERNABE, E., DEL CASTILLO, C. E. & FLORES-MIR, C. 2005. Intra-arch occlusal indicators of crowding in the permanent dentition. *American Journal of Orthodontics and Dentofacial Orthopedics,* 128**,** 220-225.

BERNER, H. S., LYNGSTADAAS, S. P., SPAHR, A., MONJO, M., THOMMESEN, L., DREVON, C. A., SYVERSEN, U. & RESELAND, J. E. 2004. Adiponectin and its receptors are expressed in bone-forming cells. *Bone,* 35**,** 842-849.

BERTL, M. H., ONODERA, K. & CELAR, A. G. 2012. A prospective randomized split-mouth study on pain experience during chairside archwire manipulation in self-ligating and conventional brackets. *The Angle Orthodontist,* 83**,** 292-297.

BHARDWAJ, S., MISRA, A., KHURANA, L., GULATI, S., SHAH, P. & VIKRAM, N. K. 2008. Childhood obesity in Asian Indians: a burgeoning cause of insulin resistance, diabetes and sub-clinical inflammation. *Asia Pacific Journal of Clinical Nutrition* 17**,** 172-175.

BIGG, H. F., SHI, Y. E., LIU, Y. E., STEFFENSEN, B. & OVERALL, C. M. 1997. Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP-4) to the COOH-terminal hemopexin-like domain of human gelatinase A TIMP-4 binds progelatinase A and the COOH-terminal domain in a similar manner to TIMP-2. *Journal of Biological Chemistry,* 272**,** 15496-15500.

BILDT, M., BLOEMEN, M., KUIJPERS-JAGTMAN, A. & VON DEN HOFF, J. 2009. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in gingival crevicular fluid during orthodontic tooth movement. *The European Journal of Orthodontics,* 31**,** 529-535.

BILDT, M., HENNEMAN, S., MALTHA, J., KUIJPERS-JAGTMAN, A. & VON DEN HOFF, J. 2007. CMT-3 inhibits orthodontic tooth displacement in the rat. *archives of oral biology,* 52**,** 571-578.

BLACK, S., KUSHNER, I. & SAMOLS, D. 2004. C-reactive protein. *Journal of Biological Chemistry,* 279**,** 48487-48490.

BLAKE, G. & RIDKER, P. 2002. Inflammatory bio-markers and cardiovascular risk prediction. *Journal of internal medicine,* 252**,** 283-294.

BLAND, J. M. & ALTMAN, D. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *The lancet,* 327**,** 307-310.

BLUHER, M., FASSHAUER, M., TöNJES, A., KRATZSCH, J., SCHöN, M. & PASCHKE, R. 2005. Association of interleukin-6, C-reactive protein, interleukin-10 and adiponectin plasma concentrations with measures of obesity, insulin sensitivity and glucose metabolism. *Experimental and clinical endocrinology & diabetes,* 113**,** 534-537.

BOGAARD, H. J., NATARAJAN, R., MIZUNO, S., ABBATE, A., CHANG, P. J., CHAU, V. Q., HOKE, N. N., KRASKAUSKAS, D., KASPER, M. & SALLOUM, F. N. 2010. Adrenergic receptor blockade reverses right heart remodeling and dysfunction in pulmonary hypertensive rats. *American journal of respiratory and critical care medicine,* 182**,** 652-660.

BOKAREWA, M., NAGAEV, I., DAHLBERG, L., SMITH, U. & TARKOWSKI, A. 2005. Resistin, an adipokine with potent proinflammatory properties. *The Journal of Immunology,* 174**,** 5789-5795.

BONAFE-OLIVEIRA, L., FALTIN, R. M. & ARANA-CHAVEZ, V. E. 2003. Ultrastructural and histochemical examination of alveolar bone at the pressure areas of rat molars submitted to continuous orthodontic force. *European journal of oral sciences,* 111**,** 410-416.

BONDEMARK, L., FREDRIKSSON, K. & ILROS, S. 2004. Separation effect and perception of pain and discomfort from two types of orthodontic separators. *World journal of orthodontics,* 5.

BOOTH, F. A., EDELMAN, J. M. & PROFFIT, W. R. 2008. Twenty-year follow-up of patients with permanently bonded mandibular canine-to-canine retainers. *American Journal of Orthodontics and Dentofacial Orthopedics,* 133**,** 70-76.

BORATO, D., PARABOCZ, G., RIBAS, J., NETTO, H., ERDMANN, F., WIECHETECK, L., MANENTE, F., DE ALMEIDA MELLO, L., BELLó, C. & DOS SANTOS, F. A. 2016. Biomarkers in obesity: serum myeloperoxidase and traditional cardiac risk parameters. *Experimental and Clinical Endocrinology & Diabetes,* 124**,** 49-54.

BORNSTEIN, S., LICINIO, J., TAUCHNITZ, R., ENGELMANN, L., NEGRAO, A., GOLD, P. & CHROUSOS, G. 1998. Plasma leptin levels are increased in survivors of acute sepsis: associated loss of diurnal rhythm in cortisol and leptin secretion. *The Journal of Clinical Endocrinology & Metabolism,* 83**,** 280-283.

BOUCHER, N. E., HANRAHAN, J. J. & KIHARA, F. Y. 1967. Occurrence of C-reactive protein in oral disease. *Journal of dental research,* 46**,** 624-624.

BOURBOULIA, D. & STETLER-STEVENSON, W. G. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. Seminars in cancer biology, 2010. Elsevier, 161-168.

BOYCE, B. F. & XING, L. 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Archives of biochemistry and biophysics,* 473**,** 139-146.

BOZKURT, F. Y., YETKIN AY, Z., SUTCU, R., DELIBAS, N. & DEMIREL, R. 2006. Gingival crevicular fluid leptin levels in periodontitis patients with long-term and heavy smoking. *Journal of periodontology,* 77**,** 634-640.

BRADLEY, R. L., ELLIS, P. E., THOMAS, P., BELLIS, H., IRELAND, A. J. & SANDY, J. R. 2007. A randomized clinical trial comparing the efficacy of ibuprofen and paracetamol in the control of orthodontic pain. *American Journal of Orthodontics and Dentofacial Orthopedics,* 132**,** 511-517.

BRENNAN, M.-L. & HAZEN, S. L. 2003. Emerging role of myeloperoxidase and oxidant stress markers in cardiovascular risk assessment. *Current opinion in lipidology,* 14**,** 353-359.

BROWN, D. F. & MOERENHOUT, R. G. 1991. The pain experience and psychological adjustment to orthodontic treatment of preadolescents, adolescents, and adults. *American Journal of Orthodontics and Dentofacial Orthopedics,* 100**,** 349-356.

BROWNLEE, M. 1992. Glycation products and the pathogenesis of diabetic complications. *Diabetes care,* 15**,** 1835-1843.

BRUUN, J. M., VERDICH, C., TOUBRO, S., ASTRUP, A. & RICHELSEN, B. 2003. Association between measures of insulin sensitivity and circulating levels of interleukin-8, interleukin-6 and tumor necrosis factor-alpha. Effect of weight loss in obese men. *European Journal of Endocrinology,* 148**,** 535-542.

BUDUNELI, N., BUDUNELI, E. & KUTUKCULER, N. 2009. Interleukin-17, RANKL, and osteoprotegerin levels in gingival crevicular fluid from smoking and non-smoking patients with chronic periodontitis during initial periodontal treatment. *Journal of periodontology,* 80**,** 1274-1280.

BURSTONE, C. J. 1962. The biomechanics of tooth movement. *Vistas in orthodontics.* Lea & Febiger, Philadelphia.

BURTIS, C. A., ASHWOOD, E. R. & BRUNS, D. E. 2012. *Tietz textbook of clinical chemistry and molecular diagnostics,* Elsevier Health Sciences.

BUTTITTA, M., ILIESCU, C., ROUSSEAU, A. & GUERRIEN, A. 2014. Quality of life in overweight and obese children and adolescents: a literature review. *Quality of life research,* 23**,** 1117-1139.

CALISKAN, M., GULLU, H., YILMAZ, S., ERDOGAN, D., UNLER, G., CIFTCI, O., TOPCU, S., CALKIN, A., ALLEN, T. & LASSILA, M. 2007. Cabré, A., I. Lázaro, J. Girona, JM Manzanares, F. Marimón, N. Plana, M. Heras, L. Masana, Fatty acid binding protein 4 is increased in metabolic syndrome and with thiazolidinedione treatment in diabetic patients 195, e150 Cahill, PA, see Cullen, JP 195, e125. *Atherosclerosis,* 195**,** 423-431.

CAMPOS, M. J. D. S., RAPOSO, N. R. B., FERREIRA, A. P. & VITRAL, R. W. F. 2011. Salivary Alpha-Amylase Activity: A Possible Indicator of Pain-Induced Stress in Orthodontic Patients. *Pain Medicine,* 12**,** 1162-1166.

CANAVAN, B., SALEM, R. O., SCHURGIN, S., KOUTKIA, P., LIPINSKA, I., LAPOSATA, M. & GRINSPOON, S. 2005. Effects of physiological leptin administration on markers of inflammation, platelet activation, and platelet aggregation during caloric deprivation. *The Journal of Clinical Endocrinology & Metabolism,* 90**,** 5779-5785.

CANAVARRO, C., TELES, R. P. & JúNIOR, J. C. 2013. Matrix metalloproteinases-1,-2,-3,-7,-8,-12, and-13 in gingival crevicular fluid during orthodontic tooth movement: a longitudinal randomized split-mouth study. *The European Journal of Orthodontics,* 35**,** 652-658.

CANTARELLA, G., CANTARELLA, R., CALTABIANO, M., RISUGLIA, N., BERNARDINI, R. & LEONARDI, R. 2006. Levels of matrix metalloproteinases 1 and 2 in human gingival crevicular fluid during initial tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 130**,** 568. e11-568. e16.

CAO, C. & SMITH, Q. 1989. Crevicular fluid myeloperoxidase at healthy, gingivitis and periodontitis sites. *Journal of clinical periodontology,* 16**,** 17-20.

CAO, J. J. 2011. Effects of obesity on bone metabolism. *Journal of orthopaedic surgery and research,* 6**,** 1.

CARBONE, F., LA ROCCA, C. & MATARESE, G. 2012. Immunological functions of leptin and adiponectin. *Biochimie,* 94**,** 2082-2088.

CARDAROPOLI, D. & GAVEGLIO, L. The influence of orthodontic movement on periodontal tissues level. Seminars in Orthodontics, 2007. Elsevier, 234-245.

CARNIELLI, V. P., LUIJENDIJK, I. H., VAN GOUDOEVER, J. B., SULKERS, E. J., BOERLAGE, A. A., DEGENHART, H. J. & SAUER, P. J. 1996. Structural position and amount of palmitic acid in infant formulas: effects on fat, fatty acid, and mineral balance. *Journal of pediatric gastroenterology and nutrition,* 23**,** 553-560.

CASA, M. A., FALTIN, R. M., FALTIN, K. & ARANA-CHAVEZ, V. E. 2006. Root resorption on torqued human premolars shown by tartrate-resistant acid phosphatase histochemistry and transmission electron microscopy. *The Angle orthodontist,* 76**,** 1015-1021.

CHAFFEE, B. W. & WESTON, S. J. 2010. Association between chronic periodontal disease and obesity: a systematic review and meta-analysis. *Journal of periodontology,* 81**,** 1708-1724.

CHAMPAGNE, C. M., BUCHANAN, W., REDDY, M. S., PREISSER, J. S., BECK, J. D. & OFFENBACHER, S. 2003. Potential for gingival crevice fluid measures as predictors of risk for periodontal diseases. *Periodontology 2000,* 31**,** 167-180.

CHAPPLE, I. L., SOCRANSKY, S. S., DIBART, S., GLENWRIGHT, D. H. & MATTHEWS, J. B. 1996. Chemiluminescent assay of alkaline phosphatase in human gingival crevicular fluid: investigations with an experimental gingivitis model and studies on the source of the enzyme within crevicular fluid. *Journal of clinical periodontology,* 23**,** 587-594.

CHARLES, P., ERIKSEN, E., MOSEKILDE, L., MELSEN, F. & JENSEN, F. 1987. Bone turnover and balance evaluated by a combined calcium balance and 47 calcium kinetic study and dynamic histomorphometry. *Metabolism,* 36**,** 1118-1124.

CHIAPPIN, S., ANTONELLI, G., GATTI, R. & DE PALO, E. F. 2007. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clinica Chimica Acta,* 383**,** 30-40.

CHRISTODOULIDES, N., FLORIANO, P. N., MILLER, C. S., EBERSOLE, J. L., MOHANTY, S., DHARSHAN, P., GRIFFIN, M., LENNART, A., BALLARD, K. L. M. & KING, C. P. 2007. Lab-on-a-chip methods for point-of-care measurements of salivary biomarkers of periodontitis. *Annals of the New York Academy of Sciences,* 1098**,** 411-428.

CIMASONI, G. 1982. Crevicular fluid updated. *Monographs in Oral Science,* 12**,** III-VII, 1-152.

CLARK, E., NESS, A. & TOBIAS, J. 2006. Adipose tissue stimulates bone growth in prepubertal children. *The Journal of Clinical Endocrinology & Metabolism,* 91**,** 2534-2541.

CONSIDINE, R. V., SINHA, M. K., HEIMAN, M. L., KRIAUCIUNAS, A., STEPHENS, T. W., NYCE, M. R., OHANNESIAN, J. P., MARCO, C. C., MCKEE, L. J. & BAUER, T. L. 1996. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine,* 334**,** 292-295.

COSTA, P. P., TREVISAN, G. L., MACEDO, G. O., PALIOTO, D. B., SOUZA, S. L., GRISI, M. F., NOVAES JR, A. B. & TABA JR, M. 2010. Salivary interleukin-6, matrix metalloproteinase-8, and osteoprotegerin in patients with periodontitis and diabetes. *Journal of periodontology,* 81**,** 384-391.

CRUMLEY, P. J. 1964. Collagen formation in the normal and stressed periodontium. *Periodontics,* 2**,** 53-61.

CRUZ, D. R., KOHARA, E. K., RIBEIRO, M. S. & WETTER, N. U. 2004. Effects of low-intensity laser therapy on the orthodontic movement velocity of human teeth: A preliminary study. *Lasers in surgery and medicine,* 35**,** 117-120.

CURTIS, M., GRIFFITHS, G., PRICE, S., COULTHURST, S. & JOHNSON, N. 1988. The total protein concentration of gingival crevicular fluid. *Journal of Clinical Periodontology,* 15**,** 628-632.

D'AIUTO, F., PARKAR, M., ANDREOU, G., SUVAN, J., BRETT, P. M., READY, D. & TONETTI, M. S. 2004. Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. *Journal of Dental Research,* 83**,** 156-160.

DALAIE, K., HAMEDI, R., KHARAZIFARD, M. J., MAHDIAN, M. & BAYAT, M. 2015. Effect of Low-Level Laser Therapy on Orthodontic Tooth Movement: A Clinical Investigation. *Journal of Dentistry (Tehran, Iran),* 12**,** 249.

DANESH, J., COLLINS, R., APPLEBY, P. & PETO, R. 1998. Association of fibrinogen, C-reactive protein, albumin, or leukocyte count with coronary heart disease: meta-analyses of prospective studies. *The Journal of the American Medical Association,* 279**,** 1477-1482.

DANESH, J., KAPTOGE, S., MANN, A. G., SARWAR, N., WOOD, A., ANGLEMAN, S. B., WENSLEY, F., HIGGINS, J. P., LENNON, L. & EIRIKSDOTTIR, G. 2008. Long-term interleukin-6 levels and subsequent risk of coronary heart disease: two new prospective studies and a systematic review. *PLoS Med,* 5**,** e78.

DANIELE, A., CAMMARATA, R., MASULLO, M., NERONE, G., FINAMORE, F., D'ANDREA, M., PILLA, F. & ORIANI, G. 2008a. Analysis of adiponectin gene and comparison of its expression in two different pig breeds. *Obesity,* 16**,** 1869-1874.

DANIELE, A., CAMMARATA, R., PASANISI, F., FINELLI, C., SALVATORI, G., CALCAGNO, G., BRACALE, R., LABRUNA, G., NARDELLI, C. & BUONO, P. 2008b. Molecular analysis of the adiponectin gene in severely obese patients from southern Italy. *Annals of Nutrition and Metabolism,* 53**,** 155-161.

DAVID, F., FARLEY, J., HUANG, H., LAVOIE, J. P. & LAVERTY, S. 2007a. Cytokine and Chemokine Gene Expression of IL-1β Stimulated Equine Articular Chondrocytes. *Veterinary surgery,* 36**,** 221-227.

DAVID, V., MARTIN, A., LAFAGE-PROUST, M.-H., MALAVAL, L., PEYROCHE, S., JONES, D. B., VICO, L. & GUIGNANDON, A. 2007b. Mechanical loading down-regulates peroxisome proliferator-activated receptor γ in bone marrow stromal cells and favors osteoblastogenesis at the expense of adipogenesis. *Endocrinology,* 148**,** 2553-2562.

DAVIDOVITCH, Z. 1995. Cell biology associated with orthodontic tooth movement. *The periodontal ligament in health and disease. St Louis: Mosby*.

DAVIDOVITCH, Z., NICOLAY, O., ALLEY, K., ZWILLING, B., LANESE, R. & SHANFELD, J. 1989. First and second messenger interactions in stressed connective tissues in vivo. *The biology of tooth movement.* CRC Press, Boca Raton, Florida.

DAVIDOVITCH, Z., NICOLAY, O., NGAN, P. & SHANFELD, J. 1988. Neurotransmitters, cytokines, and the control of alveolar bone remodeling in orthodontics. *Dental Clinics of North America,* 32**,** 411-435.

DAVIDOVITCH, Z. E. & KRISHNAN, V. 2009. Role of basic biological sciences in clinical orthodontics: a case series. *American Journal of Orthodontics and Dentofacial Orthopedics,* 135**,** 222-231.

DE ALMEIDA, P. D. V., GREGIO, A., MACHADO, M., DE LIMA, A. & AZEVEDO, L. R. 2008. Saliva composition and functions: a comprehensive review. *The Journal of Contemporary Dental Practice,* 9**,** 72-80.

DE ROSA, A., MONACO, M. L., CAPASSO, M., FORESTIERI, P., PILONE, V., NARDELLI, C., BUONO, P. & DANIELE, A. 2013. Adiponectin oligomers as potential indicators of adipose tissue improvement in obese subjects. *European Journal of Endocrinology,* 169**,** 37-43.

DEL MAR BIBILONI, M., PONS, A. & TUR, J. A. 2013. Defining body fatness in adolescents: a proposal of the AFAD-A classification. *PLoS One,* 8**,** e55849.

DELPORTE, C. 2013. Role of aquaporins in saliva secretion. *OA biochemistry*.

DENG, T., LYON, C. J., BERGIN, S., CALIGIURI, M. A. & HSUEH, W. A. 2016. Obesity, Inflammation, and Cancer. *Annual Review of Pathology: Mechanisms of Disease,* 11**,** 421-449.

DERSOT, J.-M. 2012. Gingival recession and adult orthodontics: a clinical evidence-based treatment proposal. *International Orthodontics,* 10**,** 29-42.

DEURENBERG, P., DEURENBERG YAP, M., WANG, J., LIN, F. & SCHMIDT, G. 1999. The impact of body build on the relationship between body mass index and percent body fat. *International journal of obesity,* 23**,** 537-542.

DIBIASE, A. T., NASR, I. H., SCOTT, P. & COBOURNE, M. T. 2011. Duration of treatment and occlusal outcome using Damon3 self-ligated and conventional orthodontic bracket systems in extraction patients: a prospective randomized clinical trial. *American Journal of Orthodontics and Dentofacial Orthopedics,* 139**,** e111-e116.

DILSIZ, A., KILIC, N., AYDIN, T., ATES, F. N., ZIHNI, M. & BULUT, C. 2010. Leptin levels in gingival crevicular fluid during orthodontic tooth movement. *The Angle Orthodontist,* 80**,** 504-508.

DIONNE, R. A. & BERTHOLD, C. W. 2001. Therapeutic uses of non-steroidal anti-inflammatory drugs in dentistry. *Critical Reviews in Oral Biology & Medicine,* 12**,** 315-330.

DOLCE, C., KINNIBURGH, A. J. & DZIAK, R. 1996. Immediate early-gene induction in rat osteoblastic cells after mechanical deformation. *Archives of oral biology,* 41**,** 1101-1108.

DOLCE, C., MALONE, J. S. & WHEELER, T. T. Current concepts in the biology of orthodontic tooth movement. Seminars in orthodontics, 2002. Elsevier, 6-12.

DOLL, G. M., ZENTNER, A., KLAGES, U. & SERGL, H. G. 2000. Relationship between patient discomfort, appliance acceptance and compliance in orthodontic therapy. *Journal of Orofacial Orthopedics/Fortschritte der Kieferorthopädie,* 61**,** 398-413.

DOWES, C. 2012. *Factors influencing salivary flow rate and composition. In saliva and oral health. By Edgar W.M., Dawes C. and O'Mullane D.M,* UK, Stephen Hancocks Limited.

DREBER, H., REYNISDOTTIR, S., ANGELIN, B. & HEMMINGSSON, E. 2015. Who is the Treatment-Seeking Young Adult with Severe Obesity: A Comprehensive Characterization with Emphasis on Mental Health. *PLoS One,* 10**,** e0145273.

DRUMMOND, S., CANAVARRO, C., PERINETTI, G., TELES, R. & CAPELLI, J. 2011. The monitoring of gingival crevicular fluid volume during orthodontic treatment: a longitudinal randomized split-mouth study. *The European Journal of Orthodontics***,** cjq172.

DU CLOS, T. W. 2000. Function of C-reactive protein. *Annals of medicine,* 32**,** 274-278.

DUDIC, A., KILIARIDIS, S., MOMBELLI, A. & GIANNOPOULOU, C. 2006. Composition changes in gingival crevicular fluid during orthodontic tooth movement: comparisons between tension and compression sides. *European journal of oral sciences,* 114**,** 416-422.

DUZAGAC, E., CIFCIBASI, E., ERDEM, M., KARABEY, V., KASALI, K., BADUR, S. & CINTAN, S. 2015. Is obesity associated with healing after non-surgical periodontal therapy? A local vs. systemic evaluation. *Journal of periodontal research*.

EBERSOLE, J. L. 2003. Humoral immune responses in gingival crevice fluid: local and systemic implications. *Periodontology 2000,* 31**,** 135-166.

EBERSOLE, J. L., SCHUSTER, J. L., STEVENS, J., DAWSON III, D., KRYSCIO, R. J., LIN, Y., THOMAS, M. V. & MILLER, C. S. 2013. Patterns of salivary analytes provide diagnostic capacity for distinguishing chronic adult periodontitis from health. *Journal of clinical immunology,* 33**,** 271-279.

EBRAHIMI, M., HEIDARI-BAKAVOLI, A. R., SHOEIBI, S., MIRHAFEZ, S. R., MOOHEBATI, M., ESMAILY, H., GHAZAVI, H., SABERI KARIMIAN, M.,

PARIZADEH, S. M. R. & MOHAMMADI, M. 2016. Association of Serum hs-CRP Levels With the Presence of Obesity, Diabetes Mellitus, and Other Cardiovascular Risk Factors. *Journal of clinical laboratory analysis*.

ECKEL, R. H., GRUNDY, S. M. & ZIMMET, P. Z. 2005. The metabolic syndrome. *The Lancet,* 365**,** 1415-1428.

EDGAR, W. 1992. Saliva: its secretion, composition and functions. *British dental journal,* 172**,** 305-312.

EDGAR, W., O'MULLANE, D. & DAWES, C. 2004. *Saliva and oral health*, British Dental Association London.

EKUNI, D., YAMAMOTO, T., KOYAMA, R., TSUNEISHI, M., NAITO, K. & TOBE, K. 2008. Relationship between body mass index and periodontitis in young Japanese adults. *Journal of Periodontal Research,* 43**,** 417-421.

ELIASSON, L., BIRKHED, D., ÖSTERBERG, T. & CARLEN, A. 2006. Minor salivary gland secretion rates and immunoglobulin A in adults and the elderly. *European journal of oral sciences,* 114**,** 494-499.

ELLULU, M. S., KHAZA'AI, H., RAHMAT, A., PATIMAH, I. & ABED, Y. 2016. Obesity can predict and promote systemic inflammation in healthy adults. *International journal of cardiology,* 215**,** 318-324.

EPSTEIN, L. H., PALUCH, R. & COLEMAN, K. J. 1996. Differences in salivation to repeated food cues in obese and nonobese women. *Psychosomatic Medicine,* 58**,** 160-164.

ERDINC, A. M. E. & DINCER, B. 2004. Perception of pain during orthodontic treatment with fixed appliances. *The European Journal of Orthodontics,* 26**,** 79-85.

ERIKSEN, E., MOSEKILDE, L. & MELSEN, F. 1986. Trabecular bone remodeling and balance in primary hyperparathyroidism. *Bone,* 7**,** 213-221.

ERIKSEN, E. F. 2010. Cellular mechanisms of bone remodeling. *Reviews in Endocrine and Metabolic Disorders,* 11**,** 219-227.

EUGENEROBERTS, W. & CHASE, D. C. 1981. Kinetics of cell proliferation and migration associated with orthodontically-induced osteogenesis. *Journal of Dental Research,* 60**,** 174-181.

EXLEY, M. A., HAND, L., O'SHEA, D. & LYNCH, L. 2014. Interplay between the immune system and adipose tissue in obesity. *Journal of Endocrinology,* 223**,** R41-R48.

FABIAN, T., FEJERDY, P. & CSERMELY, P. 2008. Salivary genomics, transcriptomics and proteomics: the emerging concept of the oral ecosystem and their use in the early diagnosis of cancer and other diseases. *Current genomics,* 9**,** 11-21.

FáBIáN, T. K., FEJERDY, P., NGUYEN, M. T., SŐTI, C. & CSERMELY, P. 2007. Potential immunological functions of salivary Hsp70 in mucosal and periodontal defense mechanisms. *Archivum immunologiae et therapiae experimentalis,* 55**,** 91-98.

FADEL, H. T., PLIAKI, A., GRONOWITZ, E., MåRILD, S., RAMBERG, P., DAHLEN, G., YUCEL-LINDBERG, T., HEIJL, L. & BIRKHED, D. 2013. Clinical and biological indicators of dental caries and periodontal disease in adolescents with or without obesity. *Clinical oral investigations***,** 1-10.

FAIN, J. N. 2006. Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. *Vitamins & Hormones,* 74**,** 443-477.

FALAGAS, M. E. & KOMPOTI, M. 2006. Obesity and infection. *The Lancet Infectious Diseases,* 6**,** 438-446.

FALTIN, R., FALTIN, K., SANDER, F. & ARANA-CHAVEZ, V. 2001. Ultrastructure of cementum and periodontal ligament after continuous intrusion in humans: a transmission electron microscopy study. *The European Journal of Orthodontics,* 23**,** 35-49.

FANTUZZI, G. 2005. Adipose tissue, adipokines, and inflammation. *Journal of Allergy and Clinical Immunology,* 115**,** 911-919.

FANTUZZI, G. & FAGGIONI, R. 2000. Leptin in the regulation of immunity, inflammation, and hematopoiesis. *Journal of leukocyte biology,* 68**,** 437-446.

FAROOQI, I. S., KEOGH, J. M., YEO, G. S., LANK, E. J., CHEETHAM, T. & O'RAHILLY, S. 2003. Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *New England Journal of Medicine,* 348**,** 1085-1095.

FAT, L. N. 2014. Children's body mass index, overweight and obesity. *Health and safety statistics.*

FELSON, D. T., ZHANG, Y., HANNAN, M. T. & ANDERSON, J. J. 1993. Effects of weight and body mass index on bone mineral density in men and women: the Framingham study. *Journal of Bone and Mineral Research,* 8**,** 567-573.

FENOLI-PALOMARES, C., MUñOZ-MONTAGUD, J., SANCHIZ, V., HERREROS, B., HERNáNDEZ, V., MINGUEZ, M. & BENAGES, A. 2004. Unstimulated salivary flow rate, pH and buffer capacity of saliva in healthy volunteers. *Revista espanola de enfermedades digestivas,* 96**,** 773-783.

FERNANDES, L. M., ØGAARD, B. & SKOGLUND, L. 1998. Pain and discomfort experienced after placement of a conventional or a superelastic NiTi aligning archwire. *Journal of Orofacial Orthopedics/Fortschritte der Kieferorthopädie,* 59**,** 331-339.

FIRESTONE, A. R., SCHEURER, P. A. & BURGIN, W. B. 1999. Patients' anticipation of pain and pain-related side effects, and their perception of pain as a result of orthodontic treatment with fixed appliances. *The European Journal of Orthodontics,* 21**,** 387-396.

FISHER, M. A., WENGER, R. M. & HANS, M. G. 2010. Pretreatment characteristics associated with orthodontic treatment duration. *American Journal of Orthodontics and Dentofacial Orthopedics,* 137**,** 178-186.

FITZSIMMONS, T. R., SANDERS, A. E., BARTOLD, P. M. & SLADE, G. D. 2010. Local and systemic biomarkers in gingival crevicular fluid increase odds of periodontitis. *Journal of clinical periodontology,* 37**,** 30-36.

FITZSIMMONS, T. R., SANDERS, A. E., SLADE, G. D. & BARTOLD, P. 2009. Biomarkers of periodontal inflammation in the Australian adult population. *Australian dental journal,* 54**,** 115-122.

FLEMING, P., DIBIASE, A., SARRI, G. & LEE, R. 2009. Pain experience during initial alignment with a self-ligating and a conventional fixed orthodontic appliance system: a randomized controlled clinical trial. *The Angle orthodontist,* 79**,** 46-50.

FLEMING, P. S., DIBIASE, A. T. & LEE, R. T. 2010. Randomized clinical trial of orthodontic treatment efficiency with self-ligating and conventional fixed orthodontic appliances. *American Journal of Orthodontics and Dentofacial Orthopedics,* 137**,** 738-742.

FLINK, H., BERGDAHL, M., TEGELBERG, Å., ROSENBLAD, A. & LAGERLöF, F. 2008. Prevalence of hyposalivation in relation to general health, body mass index and remaining teeth in different age groups of adults. *Community dentistry and oral epidemiology,* 36**,** 523-531.

FLINK, H., TEGELBERG, Å. & LAGERLöF, F. 2005. Influence of the time of measurement of unstimulated human whole saliva on the diagnosis of hyposalivation. *Archives of Oral Biology,* 50**,** 553-559.

FLODMARK, C.-E., LISSAU, I., MORENO, L., PIETROBELLI, A. & WIDHALM, K. 2004. New insights into the field of children and adolescents' obesity: the European perspective. *International journal of obesity,* 28**,** 1189-1196.

FLORES, L. S., GAYA, A. R., PETERSEN, R. D. & GAYA, A. 2013. Trends of underweight, overweight, and obesity in Brazilian children and adolescents. *Jornal de pediatria,* 89**,** 456-461.

FLóREZ-MORENO, G. A., ISAZA-GUZMáN, D. M. & TOBóN-ARROYAVE, S. I. 2013a. Time-related changes in salivary levels of the osteotropic factors sRANKL and OPG through orthodontic tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 143**,** 92-100.

FLóREZ-MORENO, G. A., MARíN-RESTREPO, L. M., ISAZA-GUZMáN, D. M. & TOBóN-ARROYAVE, S. I. 2013b. Screening for salivary levels of deoxypyridinoline and bone-specific alkaline phosphatase during orthodontic tooth movement: a pilot study. *The European Journal of Orthodontics,* 35**,** 361-368.

FORSBERG, C.-M., OLIVEBY, A. & LAGERLöF, F. 1992. Salivary clearance of sugar before and after insertion of fixed orthodontic appliances. *American Journal of Orthodontics and Dentofacial Orthopedics,* 102**,** 527-530.

FRAZIER-BOWERS, S. A., SIMMONS, D., WRIGHT, J. T., PROFFIT, W. R. & ACKERMAN, J. L. 2010. Primary failure of eruption and PTH1R: the importance of a genetic diagnosis for orthodontic treatment planning. *American Journal of Orthodontics and Dentofacial Orthopedics,* 137**,** 160. e1-160. e7.

FREEDMAN, D. S. & SHERRY, B. 2009. The validity of BMI as an indicator of body fatness and risk among children. *Pediatrics,* 124**,** S23-S34.

FRENCH, S. A., STORY, M. & PERRY, C. L. 1995. Self-esteem and obesity in children and adolescents: a literature review. *Obesity research,* 3**,** 479-490.

FRIEDMAN, J. M. 2000. Obesity in the new millennium. *Nature,* 404**,** 632-634.

FRODGE, B. D., EBERSOLE, J. L., KRYSCIO, R. J., THOMAS, M. V. & MILLER, C. S. 2008. Bone remodeling biomarkers of periodontal disease in saliva. *Journal of periodontology,* 79**,** 1913-1919.

FUERSTENBERG, R., ANDERSON, S., BRUMBAUGH, K. & SCHMIDT, J. 2014. Multiplex analysis of secreted biomarkers from stimulated natural killer cells (INC6P. 337). *The Journal of Immunology,* 192**,** 121.4-121.4.

FUHLBRIGGE, R. C., KIEFFER, J. D., ARMERDING, D. & KUPPER, T. S. 1997. Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. *Nature,* 389**,** 978-981.

FULLER, K., GALLAGHER, A. & CHAMBERS, T. 1991. Osteoclast resorption—stimulating activity is associated with the osteoblast cell surface and/or the extracellular matrix. *Biochemical and biophysical research communications,* 181**,** 67-73.

FUNAHASHI, T., NAKAMURA, T., SHIMOMURA, I., MAEDA, K., KURIYAMA, H., TAKAHASHI, M., ARITA, Y., KIHARA, S. & MATSUZAWA, Y. 1999. Role of adipocytokines on the pathogenesis of atherosclerosis in visceral obesity. *Internal medicine (Tokyo, Japan),* 38**,** 202-206.

FURUGEN, R., HAYASHIDA, H., YAMAGUCHI, N., YOSHIHARA, A., OGAWA, H., MIYAZAKI, H. & SAITO, T. 2008. The relationship between periodontal condition

and serum levels of resistin and adiponectin in elderly Japanese. *Journal of periodontal research,* 43**,** 556-562.

GABAY, C. 2006. Interleukin-6 and chronic inflammation. *Arthritis research and therapy,* 8**,** S3.

GALLER, A., GELBRICH, G., KRATZSCH, J., NOACK, N., KAPELLEN, T. & KIESS, W. 2007. Elevated serum levels of adiponectin in children, adolescents and young adults with type 1 diabetes and the impact of age, gender, body mass index and metabolic control: a longitudinal study. *European Journal of Endocrinology,* 157**,** 481-489.

GARG, A. K. 2009. *Implant dentistry*, Elsevier Health Sciences.

GENCO, R. J. 1996. Current View of Risk Factors for Periodontal Diseases*. *Journal of periodontology,* 67**,** 1041-1049.

GENCO, R. J. 2012. Salivary diagnostic tests. *The Journal of the American Dental Association,* 143**,** 3S-5S.

GENCO, R. J. & BORGNAKKE, W. S. 2013. Risk factors for periodontal disease. *Periodontology 2000,* 62**,** 59-94.

GEOFFROY, V., MARTY-MORIEUX, C., LE GOUPIL, N., CLEMENT-LACROIX, P., TERRAZ, C., FRAIN, M., ROUX, S., ROSSERT, J. & DE VERNEJOUL, M. C. 2004. In vivo inhibition of osteoblastic metalloproteinases leads to increased trabecular bone mass. *Journal of Bone and Mineral Research,* 19**,** 811-822.

GEORGE, A. & EVANS, C. 2009. Detection of root resorption using dentin and bone markers. *Orthodontics & craniofacial research,* 12**,** 229-235.

GIANNOBILE, W. V. 2012. Salivary diagnostics for periodontal diseases. *The Journal of the American Dental Association,* 143**,** 6S-11S.

GIANNOPOULOU, C., DI FELICE, R., ANDERSEN, E. & CIMASONI, G. 1990. Synthesis of α2-macroglobulin in human gingiva: A study of the concentration of macroglobulin and albumin in gingival fluid and serum. *Archives of oral biology,* 35**,** 13-16.

GIANNOPOULOU, C., DUDIC, A. & KILIARIDIS, S. 2006. Pain discomfort and crevicular fluid changes induced by orthodontic elastic separators in children. *The Journal of Pain,* 7**,** 367-376.

GIBBINS, H., PROCTOR, G., YAKUBOV, G., WILSON, S. & CARPENTER, G. 2014. Concentration of salivary protective proteins within the bound oral mucosal pellicle. *Oral diseases,* 20**,** 707-713.

GIMBLE, J. M., ROBINSON, C. E., WU, X., KELLY, K. A., RODRIGUEZ, B. R., KLIEWER, S. A., LEHMANN, J. M. & MORRIS, D. C. 1996. Peroxisome proliferator-activated receptor-gamma activation by thiazolidinediones induces adipogenesis in bone marrow stromal cells. *Molecular pharmacology,* 50**,** 1087-1094.

GIUCA, M. R., PASINI, M., TECCO, S., MARCHETTI, E., GIANNOTTI, L. & MARZO, G. 2012. Skeletal maturation in obese patients. *American Journal of Orthodontics and Dentofacial Orthopedics,* 142**,** 774-779.

GKANTIDIS, N., MISTAKIDIS, I., KOUSKOURA, T. & PANDIS, N. 2014. Effectiveness of non-conventional methods for accelerated orthodontic tooth movement: a systematic review and meta-analysis. *Journal of dentistry,* 42**,** 1300-1319.

GOKHALE, N. H., ACHARYA, A. B., PATIL, V. S., TRIVEDI, D. J., SETTY, S. & THAKUR, S. L. 2014. Resistin levels in gingival crevicular fluid of patients with

chronic periodontitis and type 2 diabetes mellitus. *Journal of periodontology,* 85**,** 610-617.

GOLDBERG, G. I., STRONGIN, A., COLLIER, I., GENRICH, L. & MARMER, B. 1992. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *Journal of Biological Chemistry,* 267**,** 4583-4591.

GOLUB, L. & KLEINBERG, I. 1976. Gingival crevicular fluid: a new diagnostic aid in managing the periodontal patient. *Oral Sciences Reviews***,** 49.

GOMEZ, D., ALONSO, D., YOSHIJI, H. & THORGEIRSSON, U. 1997. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *European journal of cell biology,* 74**,** 111-122.

GONCALVES, T. E. D., ZIMMERMANN, G. S., FIGUEIREDO, L. C., SOUZA, M. D. C., CRUZ, D. F., BASTOS, M. F., SILVA, H. D. P. & DUARTE, P. M. 2015. Local and serum levels of adipokines in patients with obesity after periodontal therapy: one-year follow-up. *Journal of clinical periodontology,* 42**,** 431-439.

GOODSON, J., GROPPO, D., HALEM, S. & CARPINO, E. 2009. Is obesity an oral bacterial disease? *Journal of dental research,* 88**,** 519-523.

GOODSON, J. M. 2003. Gingival crevice fluid flow. *Periodontology 2000,* 31**,** 43-54.

GOODSON, J. M., KANTARCI, A., HARTMAN, M.-L., DENIS, G. V., STEPHENS, D., HASTURK, H., YASKELL, T., VARGAS, J., WANG, X. & CUGINI, M. 2014. Metabolic disease risk in children by salivary biomarker analysis. *PloS one,* 9**,** e98799.

GORAN, M. I. 1998. Measurement issues related to studies of childhood obesity: assessment of body composition, body fat distribution, physical activity, and food intake. *Pediatrics,* 101**,** 505-518.

GORMAN, A., KAYE, E. K., APOVIAN, C., FUNG, T. T., NUNN, M. & GARCIA, R. I. 2012. Overweight and obesity predict time to periodontal disease progression in men. *Journal of clinical periodontology,* 39**,** 107-114.

GOTO, H., OSAKI, M., FUKUSHIMA, T., SAKAMOTO, K., HOZUMI, A., BABA, H. & SHINDO, H. 2011. Human bone marrow adipocytes support dexamethasone-induced osteoclast differentiation and function through RANKL expression. *Biomedical Research,* 32**,** 37-44.

GRABER, L. W., VANARSDALL JR, R. L. & VIG, K. W. 2011. *Orthodontics: current principles and techniques*, Elsevier Health Sciences.

GRANT, M., WILSON, J., ROCK, P. & CHAPPLE, I. 2013. Induction of cytokines, MMP9, TIMPs, RANKL and OPG during orthodontic tooth movement. *The European Journal of Orthodontics,* 35**,** 644-651.

GRAY, R. 2004. Occlusion and Clinical Practice—An Evidence Based Approach. *British Dental Journal,* 197**,** 107-107.

GREGOIRE, F. M., SMAS, C. M. & SUL, H. S. 1998. Understanding adipocyte differentiation. *Physiological reviews,* 78**,** 783-809.

GREQOIR, F., SMAS, C. & SUL, H. 1998. Understanding adipocyte differentaion. *Physiol Rev,* 78**,** 783-809.

GRIEVE, W. G., JOHNSON, G. K., MOORE, R. N., REINHARDT, R. A. & DUBOIS, L. M. 1994. Prostaglandin E (PGE) and interleukin-1β (IL-1β) levels in gingival crevicular fluid during human orthodontic tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 105**,** 369-374.

GRIFFITHS, G., MOULSON, A., PETRIE, A. & JAMES, I. 1998. Evaluation of osteocalcin and pyridinium crosslinks of bone collagen as markers of bone turnover in gingival crevicular fluid during different stages of orthodontic treatment. *Journal of clinical periodontology,* 25**,** 492-498.

GRIFFITHS, G., STERNE, J., WILTON, J., EATON, K. & JOHNSON, N. 1992. Associations between volume and flow rate of gingival crevicular fluid and clinical assessments of gingival inflammation in a population of British male adolescents. *Journal of clinical periodontology,* 19**,** 464-470.

GRIFFITHS, G., WILTON, J. & CURTIS, M. 1997. Permeability of the gingival tissues to IgM during an experimental gingivitis study in man. *Archives of oral biology,* 42**,** 129-136.

GRIFFITHS, G. S. 2003. Formation, collection and significance of gingival crevice fluid. *Periodontology 2000,* 31**,** 32-42.

GRÖSCHL, M., RAUH, M., WAGNER, R., NEUHUBER, W., METZLER, M., TAMGÜNEY, G. L., ZENK, J., SCHOOF, E., DÖRR, H. G. & BLUM, W. F. 2001. Identification of leptin in human saliva. *The Journal of Clinical Endocrinology & Metabolism,* 86**,** 5234-5239.

GUNELI, E., GUMUSTEKIN, M. & ATES, M. 2010. Possible involvement of ghrelin on pain threshold in obesity. *Medical hypotheses,* 74**,** 452-454.

GURSOY, U. K., KöNöNEN, E., PRADHAN-PALIKHE, P., TERVAHARTIALA, T., PUSSINEN, P. J., SUOMINEN-TAIPALE, L. & SORSA, T. 2010. Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. *Journal of clinical periodontology,* 37**,** 487-493.

GURSOY, U. K., KöNöNEN, E., UITTO, V. J., PUSSINEN, P. J., HYVäRINEN, K., SUOMINEN-TAIPALE, L. & KNUUTTILA, M. 2009. Salivary interleukin-1β concentration and the presence of multiple pathogens in periodontitis. *Journal of clinical periodontology,* 36**,** 922-927.

HADA, Y., YAMAUCHI, T., WAKI, H., TSUCHIDA, A., HARA, K., YAGO, H., MIYAZAKI, O., EBINUMA, H. & KADOWAKI, T. 2007. Selective purification and characterization of adiponectin multimer species from human plasma. *Biochemical and Biophysical Research Communications,* 356**,** 487-493.

HåHEIM, L. L., NAFSTAD, P., OLSEN, I., SCHWARZE, P. & RøNNINGEN, K. S. 2009. C-reactive protein variations for different chronic somatic disorders. *Scandinavian journal of public health,* 37**,** 640-646.

HALADE, G. V., EL JAMALI, A., WILLIAMS, P. J., FAJARDO, R. J. & FERNANDES, G. 2011. Obesity-mediated inflammatory microenvironment stimulates osteoclastogenesis and bone loss in mice. *Experimental gerontology,* 46**,** 43-52.

HALBERG, N., WERNSTEDT-ASTERHOLM, I. & SCHERER, P. E. 2008. The adipocyte as an endocrine cell. *Endocrinology and metabolism clinics of North America,* 37**,** 753-768.

HASHIZUME, L. N., BASTOS, L. F., CARDOZO, D. D., HILGERT, J. B., HUGO, F. N., STEIN, A. T., SOUTO, K. E. & MEINHARDT, N. G. 2015. Impact of Bariatric Surgery on the Saliva of Patients with Morbid Obesity. *Obesity surgery,* 25**,** 1550-1555.

HEASMAN, P. A., MILLETT, D. T. & CHAPPLE, I. L. 1996. *The periodontium and orthodontics in health and disease*, Oxford University Press, USA.

HEDIN, C., RONQUIST, G. & FORSBERG, O. 1981. Cyclic nucleotide content in gingival tissue of smokers and non-smokers. *Journal of Periodontal Research,* 16**,** 337-343.

HELMERHORST, E. J. 2012. *protective functions of saliva. In saliva and oral health, By Edgar W.M., Dawes C. and O'Mullane D.M,* UK, Stephen Hancocks Limited.

HENNEMAN, S., VON DEN HOFF, J. & MALTHA, J. 2008. Mechanobiology of tooth movement. *The European Journal of Orthodontics,* 30**,** 299-306.

HEO, M., ALLISON, D. B., FAITH, M. S., ZHU, S. & FONTAINE, K. R. 2003. Obesity and quality of life: mediating effects of pain and comorbidities. *Obesity research,* 11**,** 209-216.

HILL, P. & ORTH, M. 1998. Bone remodelling. *Journal of Orthodontics,* 25**,** 101-107.

HIROSE, H., YAMAMOTO, Y., SEINO-YOSHIHARA, Y., KAWABE, H. & SAITO, I. 2010. Serum high-molecular-weight adiponectin as a marker for the evaluation and care of subjects with metabolic syndrome and related disorders. *Journal of atherosclerosis and thrombosis,* 17**,** 1201-1211.

HOLLIDAY, L., VAKANI, A., ARCHER, L. & DOLCE, C. 2003. Effects of matrix metalloproteinase inhibitors on bone resorption and orthodontic tooth movement. *Journal of Dental Research,* 82**,** 687-691.

HOLMBECK, K., BIANCO, P., YAMADA, S. & BIRKEDAL-HANSEN, H. 2004. MT1-MMP: a tethered collagenase. *Journal of cellular physiology,* 200**,** 11-19.

HOOGEVEEN, E. J., JANSMA, J. & REN, Y. 2014. Surgically facilitated orthodontic treatment: a systematic review. *American Journal of Orthodontics and Dentofacial Orthopedics,* 145**,** S51-S64.

HUG, C., WANG, J., AHMAD, N. S., BOGAN, J. S., TSAO, T.-S. & LODISH, H. F. 2004. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 10308-10313.

HUMPHREY, S. P. & WILLIAMSON, R. T. 2001. A review of saliva: normal composition, flow, and function. *The Journal of prosthetic dentistry,* 85**,** 162-169.

IDE, M., JAGDEV, D., COWARD, P. Y., CROOK, M., BARCLAY, G. R. & WILSON, R. F. 2004. The short-term effects of treatment of chronic periodontitis on circulating levels of endotoxin, C-reactive protein, tumor necrosis factor-α, and interleukin-6. *Journal of periodontology,* 75**,** 420-428.

INGMAN, T., APAJALAHTI, S., MäNTYLä, P., SAVOLAINEN, P. & SORSA, T. 2005. Matrix metalloproteinase-1 and-8 in gingival crevicular fluid during orthodontic tooth movement: a pilot study during 1 month of follow-up after fixed appliance activation. *The European Journal of Orthodontics,* 27**,** 202-207.

INGMAN, T., TERVAHARTIALA, T., DING, Y., TSCHESCHE, H., HAERIAN, A., KINANE, D. F., KONTTINEN, Y. T. & SORSA, T. 1996. Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva of periodontitis patients. *Journal of clinical periodontology,* 23**,** 1127-1132.

IRELAND, A. J., ELLIS, P., JORDAN, A., BRADLEY, R., EWINGS, P., ATACK, N. E., GRIFFITHS, H., HOUSE, K., MOORE, M. B. & DEACON, S. 2017. Chewing gum vs. ibuprofen in the management of orthodontic pain, a multi-centre randomised controlled trial–the effect of anxiety. *Journal of orthodontics,* 44**,** 3-7.

ISHIBASHI, O., NIWA, S., KADOYAMA, K. & INUI, T. 2006. MMP-9 antisense oligodeoxynucleotide exerts an inhibitory effect on osteoclastic bone resorption by suppressing cell migration. *Life sciences,* 79**,** 1657-1660.

ISSA, R. I. & GRIFFIN, T. M. 2012. Pathobiology of obesity and osteoarthritis: integrating biomechanics and inflammation. *Pathobiology of Aging & Age-related Diseases,* 2.

IVASKA, K. K., HUOVINEN, V., SOINIO, M., HANNUKAINEN, J. C., SAUNAVAARA, V., SALMINEN, P., HELMIö, M., PARKKOLA, R., NUUTILA,

P. & KIVIRANTA, R. 2016. Changes in bone metabolism after bariatric surgery by gastric bypass or sleeve gastrectomy. *Bone*.

IWASAKI, L., CHANDLER, J., MARX, D., PANDEY, J. & NICKEL, J. 2009. IL-1 gene polymorphisms, secretion in gingival crevicular fluid, and speed of human orthodontic tooth movement. *Orthodontics & craniofacial research,* 12**,** 129-140.

IWASAKI, L., HAACK, J., NICKEL, J., REINHARDT, R. A. & PETRO, T. M. 2001. Human interleukin-1β and interleukin-1 receptor antagonist secretion and velocity of tooth movement. *Archives of Oral Biology,* 46**,** 185-189.

IWASAKI, L. R., CROUCH, L. D., TUTOR, A., GIBSON, S., HUKMANI, N., MARX, D. B. & NICKEL, J. C. 2005. Tooth movement and cytokines in gingival crevicular fluid and whole blood in growing and adult subjects. *American journal of orthodontics and dentofacial orthopedics,* 128**,** 483-491.

IWAYAMA, T., YANAGITA, M., MORI, K., SAWADA, K., OZASA, M., KUBOTA, M., MIKI, K., KOJIMA, Y., TAKEDACHI, M. & KITAMURA, M. 2012. Adiponectin regulates functions of gingival fibroblasts and periodontal ligament cells. *Journal of periodontal research,* 47**,** 563-571.

JABŁOŃSKA-TRYPUĆ, A., MATEJCZYK, M. & ROSOCHACKI, S. 2016. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. *Journal of enzyme inhibition and medicinal chemistry***,** 1-7.

JAISWAL, A., CHHABRA, A., MALHOTRA, U., KOHLI, S. & RANI, V. 2011. Comparative analysis of human matrix metalloproteinases: Emerging therapeutic targets in diseases. *Bioinformation,* 6**,** 23-30.

JANDIK, K. A., BELANGER, M., LOW, S., DORN, B., YANG, M. & PROGULSKE-FOX, A. 2008. Invasive differences among Porphyromonas gingivalis strains from healthy and diseased periodontal sites. *Journal of periodontal research,* 43**,** 524-530.

JANICKA, A., WREN, T. A., SANCHEZ, M. M., DOREY, F., KIM, P. S., MITTELMAN, S. D. & GILSANZ, V. 2007. Fat mass is not beneficial to bone in adolescents and young adults. *The Journal of Clinical Endocrinology & Metabolism,* 92**,** 143-147.

JAYE, D. L. & WAITES, K. B. 1997. Clinical applications of C-reactive protein in pediatrics. *The Pediatric infectious disease journal,* 16**,** 735-747.

JIAN, F., LAI, W., FURNESS, S., MCINTYRE, G. T., MILLETT, D. T., HICKMAN, J. & WANG, Y. 2013. Initial arch wires for tooth alignment during orthodontic treatment with fixed appliances. *The Cochrane Library*.

JIMENEZ, M., HU, F. B., MARINO, M., LI, Y. & JOSHIPURA, K. J. 2012. Prospective associations between measures of adiposity and periodontal disease. *Obesity,* 20**,** 1718-1725.

JOHAL, A., FLEMING, P. & AL JAWAD, F. 2014. A prospective longitudinal controlled assessment of pain experience and oral health-related quality of life in adolescents undergoing fixed appliance treatment. *Orthodontics & craniofacial research,* 17**,** 178-186.

JOHNSON, R. & SERIO, F. 2001. Leptin within healthy and diseased human gingiva. *Journal of Periodontology,* 72**,** 1254-1257.

JONES, M. 1984. An investigation into the initial discomfort caused by placement of an archwire. *The European Journal of Orthodontics,* 6**,** 48-54.

JONES, M. & CHAN, C. 1992. The pain and discomfort experienced during orthodntic treatment: A randomized controlled clinical trial of two intial aligning arch wires. *American Journal of Orthodontics and Dentofacial Orthopedics,* 102**,** 373-381.

JONES, M. & RICHMOND, S. 1985. Initial tooth movement: force application and pain—a relationship? *American journal of orthodontics,* 88**,** 111-116.

JOSEPH, G. Y., JAVORSCHI, S., HEVENER, A. L., KRUSZYNSKA, Y. T., NORMAN, R. A., SINHA, M. & OLEFSKY, J. M. 2002. The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes,* 51**,** 2968-2974.

JOSHI, M. B., PHILIPPOVA, M., IVANOV, D., ALLENSPACH, R., ERNE, P. & RESINK, T. J. 2005. T-cadherin protects endothelial cells from oxidative stress-induced apoptosis. *The FASEB journal,* 19**,** 1737-1739.

JUNIOR, J. C., KANTARCI, A., HAFFAJEE, A., TELES, R. P., FIDEL, R. & FIGUEREDO, C. M. 2011. Matrix metalloproteinases and chemokines in the gingival crevicular fluid during orthodontic tooth movement. *The European Journal of Orthodontics,* 33**,** 705-711.

KADOWAKI, T. & YAMAUCHI, T. 2005. Adiponectin and adiponectin receptors. *Endocrine reviews,* 26**,** 439-451.

KAJIYA, M., GIRO, G., TAUBMAN, M. A., HAN, X., MAYER, M. P. & KAWAI, T. 2010. Role of periodontal pathogenic bacteria in RANKL-mediated bone destruction in periodontal disease. *Journal of oral microbiology,* 2.

KALE, S., KOCADERELI, I. L., ATILLA, P. & AŞAN, E. 2004. Comparison of the effects of 1, 25 dihydroxycholecalciferol and prostaglandin E 2 on orthodontic tooth movement. *American journal of orthodontics and dentofacial orthopedics,* 125**,** 607-614.

KANER, D., BERNIMOULIN, J. P., KLEBER, B. M., HEIZMANN, W. R. & FRIEDMANN, A. 2006. Gingival crevicular fluid levels of calprotectin and myeloperoxidase during therapy for generalized aggressive periodontitis. *Journal of periodontal research,* 41**,** 132-139.

KANTOVITZ, K. R., PASCON, F. M., RONTANI, R. M. P. & GAVIAO, M. B. D. 2006. Obesity and dental caries-A systematic review. *Oral Health and Preventive Dentistry,* 4**,** 137.

KANZAKI, H., CHIBA, M., TAKAHASHI, I., HARUYAMA, N., NISHIMURA, M. & MITANI, H. 2004. Local OPG gene transfer to periodontal tissue inhibits orthodontic tooth movement. *Journal of Dental Research,* 83**,** 920-925.

KAPOOR, P., KHARBANDA, O. P., MONGA, N., MIGLANI, R. & KAPILA, S. 2014. Effect of orthodontic forces on cytokine and receptor levels in gingival crevicular fluid: a systematic review. *Progress in orthodontics,* 15**,** 1.

KARACAY, S., SAYGUN, I., BENGI, A. O. & SERDAR, M. 2007. Tumor Necrosis Factor-α levels during two different canine distalization techniques. *The Angle orthodontist,* 77**,** 142-147.

KARTHIKEYAN, B. & PRADEEP, A. 2007a. Gingival crevicular fluid and serum leptin: their relationship to periodontal health and disease. *Journal of clinical periodontology,* 34**,** 467-472.

KARTHIKEYAN, B. & PRADEEP, A. 2007b. Leptin levels in gingival crevicular fluid in periodontal health and disease. *Journal of periodontal research,* 42**,** 300-304.

KASER, A. & TILG, H. 2008. Novel therapeutic targets in the treatment of IBD.

KATSIOUGIANNIS, S., KAPSOGEORGOU, E. K., MANOUSSAKIS, M. N. & SKOPOULI, F. N. 2006. Salivary gland epithelial cells: a new source of the immunoregulatory hormone adiponectin. *Arthritis and Rheumatism,* 54**,** 2295-2299.

KATSIOUGIANNIS, S., TENTA, R. & SKOPOULI, F. N. 2010. Activation of AMP-activated protein kinase by adiponectin rescues salivary gland epithelial cells from

spontaneous and interferon-γ–induced apoptosis. *Arthritis and Rheumatism,* 62**,** 414-419.

KAU, C. H., KANTARCI, A., SHAUGHNESSY, T., VACHIRAMON, A., SANTIWONG, P., DE LA FUENTE, A., SKRENES, D., MA, D. & BRAWN, P. 2013. Photobiomodulation accelerates orthodontic alignment in the early phase of treatment. *Progress in orthodontics,* 14**,** 1.

KAUFMAN, E. & LAMSTER, I. B. 2000. Analysis of saliva for periodontal diagnosis. *Journal of clinical periodontology,* 27**,** 453-465.

KAUKUA, J., PEKKARINEN, T., SANE, T. & MUSTAJOKI, P. 2003. Health-related quality of life in obese outpatients losing weight with very-low-energy diet and behaviour modification—a 2-y follow-up study. *International journal of obesity,* 27**,** 1233-1241.

KAVADIA-TSATALA, S., KAKLAMANOS, E. & TSALIKIS, L. 2001. Effects of orthodontic treatment on gingival crevicular fluid flow rate and composition: clinical implications and applications. *The International journal of adult orthodontics and orthognathic surgery,* 17**,** 191-205.

KAWASAKI, K., TAKAHASHI, T., YAMAGUCHI, M. & KASAI, K. 2006. Effects of aging on RANKL and OPG levels in gingival crevicular fluid during orthodontic tooth movement. *Orthodontics & craniofacial research,* 9**,** 137-142.

KAYA, F. A., HAMAMCı, N., UYSAL, E. & YOKUŞ, B. 2011. Identification of tumor necrosis factor-α levels around miniscrews during canine distalization. *Korean Journal of Orthodontics,* 41**,** 36-41.

KELLER, A., ROHDE, J. F., RAYMOND, K. & HEITMANN, B. L. 2015. Association between periodontal disease and overweight and obesity: a systematic review. *Journal of periodontology,* 86**,** 766-776.

KHADER, Y. S., BAWADI, H. A., HAROUN, T. F., ALOMARI, M. & TAYYEM, R. F. 2009. The association between periodontal disease and obesity among adults in Jordan. *Journal of clinical periodontology,* 36**,** 18-24.

KHANNA, S. & MALI, A. M. 2010. Evaluation of tumor necrosis factor-α (TNF-α) levels in plasma and their correlation with periodontal status in obese and non-obese subjects. *Journal of Indian Society of Periodontology,* 14**,** 217.

KHAODHIAR, L., LING, P.-R., BLACKBURN, G. L. & BISTRIAN, B. R. 2004. Serum levels of interleukin-6 and C-reactive protein correlate with body mass index across the broad range of obesity. *Journal of Parenteral and Enteral Nutrition,* 28**,** 410-415.

KHOSLA, S. 2001. Minireview: The opg/rankl/rank system. *Endocrinology,* 142**,** 5050-5055.

KHOSRAVI, R., TRAN, S. D., LAMBERT, M., O'LOUGHLIN, J., KA, K., FEINE, J. S., CARON, C., TREMBLAY, A. & NICOLAU, B. 2009. Adiposity and gingival crevicular fluid tumour necrosis factor-α levels in children. *Journal of Clinical Periodontology,* 36**,** 301-307.

KINANE, D. F., SHIBA, H. & HART, T. C. 2005. The genetic basis of periodontitis. *Periodontology 2000,* 39**,** 91-117.

KINDT, T. J., GOLDSBY, R. A., OSBORNE, B. A. & KUBY, J. 2007. *Kuby immunology,* Macmillan.

KING, G., KEELING, S. & WRONSKI, T. 1991. Histomorphometric study of alveolar bone turnover in orthodontic tooth movement. *Bone,* 12**,** 401-409.

KINNEY, J., MORELLI, T., BRAUN, T., RAMSEIER, C., HERR, A., SUGAI, J., SHELBURNE, C., RAYBURN, L., SINGH, A. & GIANNOBILE, W. 2011.

Saliva/pathogen biomarker signatures and periodontal disease progression. *Journal of dental research,* 90**,** 752-758.

KINNEY, J. S., MORELLI, T., OH, M., BRAUN, T. M., RAMSEIER, C. A., SUGAI, J. V. & GIANNOBILE, W. V. 2014. Crevicular fluid biomarkers and periodontal disease progression. *Journal of Clinical Periodontology,* 41**,** 113-120.

KINNEY, J. S., RAMSEIER, C. A. & GIANNOBILE, W. V. 2007. Oral fluid–based biomarkers of alveolar bone loss in periodontitis. *Annals of the New York Academy of Sciences,* 1098**,** 230-251.

KISHORE, U., GABORIAUD, C., WATERS, P., SHRIVE, A. K., GREENHOUGH, T. J., REID, K. B., SIM, R. B. & ARLAUD, G. J. 2004. C1q and tumor necrosis factor superfamily: modularity and versatility. *Trends in immunology,* 25**,** 551-561.

KITASE, Y., YOKOZEKI, M., FUJIHARA, S., IZAWA, T., KURODA, S., TANIMOTO, K., MORIYAMA, K. & TANAKA, E. 2009. Analysis of gene expression profiles in human periodontal ligament cells under hypoxia: the protective effect of CC chemokine ligand 2 to oxygen shortage. *archives of oral biology,* 54**,** 618-624.

KNAŚ, M., MACIEJCZYK, M., SAWICKA, K., HADY, H. R., NICZYPORUK, M., ŁADNY, J. R., MATCZUK, J., WASZKIEL, D., ŻENDZIAN-PIOTROWSKA, M. & ZALEWSKA, A. 2015. Impact of morbid obesity and bariatric surgery on antioxidant/oxidant balance of the unstimulated and stimulated human saliva. *Journal of Oral Pathology & Medicine*.

KOH, D. S.-Q. & KOH, G. C.-H. 2007. The use of salivary biomarkers in occupational and environmental medicine. *Occupational and environmental medicine,* 64**,** 202-210.

KOH, K. K., HAN, S. H. & QUON, M. J. 2005. Inflammatory markers and the metabolic syndrome: insights from therapeutic interventions. *Journal of the American College of Cardiology,* 46**,** 1978-1985.

KOOK, S. H., JANG, Y. S. & LEE, J. C. 2011. Human periodontal ligament fibroblasts stimulate osteoclastogenesis in response to compression force through TNF-α-mediated activation of CD4+ T cells. *Journal of cellular biochemistry,* 112**,** 2891-2901.

KOPELMAN, P. G. 2000. Obesity as a medical problem. *Nature,* 404**,** 635-643.

KOYAMA, Y., MITSUI, N., SUZUKI, N., YANAGISAWA, M., SANUKI, R., ISOKAWA, K., SHIMIZU, N. & MAENO, M. 2008. Effect of compressive force on the expression of inflammatory cytokines and their receptors in osteoblastic Saos-2 cells. *Archives of oral biology,* 53**,** 488-496.

KRISHNAN, V. 2007. Orthodontic pain: from causes to management—a review. *The European Journal of Orthodontics,* 29**,** 170-179.

KRISHNAN, V. & DAVIDOVITCH, Z. 2006a. The effect of drugs on orthodontic tooth movement. *Orthodontics & craniofacial research,* 9**,** 163-171.

KRISHNAN, V. & DAVIDOVITCH, Z. 2009. On a path to unfolding the biological mechanisms of orthodontic tooth movement. *Journal of Dental Research,* 88**,** 597-608.

KRISHNAN, V. & DAVIDOVITCH, Z. E. 2006b. Cellular, molecular, and tissue-level reactions to orthodontic force. *American Journal of Orthodontics and Dentofacial Orthopedics,* 129**,** 469. e1-469. e32.

KRISHNAN, V. & DAVIDOVITCH, Z. E. 2015. Biologic orthodontics: Methods to accelerate or decelerate orthodontic tooth movement. *Biological Mechanisms of Tooth Movement***,** 191.

KRYSIAK, R., HANDZLIK-ORLIK, G. & OKOPIEN, B. 2012. The role of adipokines in connective tissue diseases. *European journal of nutrition,* 51**,** 513-528.

KUMAR, A. A., SARAVANAN, K., KOHILA, K. & KUMAR, S. S. 2015. Biomarkers in orthodontic tooth movement. *Journal of pharmacy & bioallied sciences,* 7**,** S325.

KUROKI, H., MIYAGAWA, Y., SHIMOMURA-KUROKI, J., ENDO, T. & SHIMOMURA, H. 2014. Identification of marker proteins by orthodontic treatment: relationship of RANKL in the gingival crevicular fluid and of amylase in whole saliva with orthodontic treatment. *Odontology,* 102**,** 303-309.

KURTIS, B., TUTER, G., SERDAR, M., AKDEMIR, P., UYGUR, C., FIRATLI, E. & BAL, B. 2005. Gingival crevicular fluid levels of monocyte chemoattractant protein-1 and tumor necrosis factor-alpha in patients with chronic and aggressive periodontitis. *Journal of periodontology,* 76**,** 1849-1855.

KUSY, R. P. & TULLOCH, J. C. 1986. Analysis of moment/force ratios in the mechanics of tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 90**,** 127-131.

KUTLU, S., CANPOLAT, S., SANDAL, S., OZCAN, M., SARSILMAZ, M. & KELESTIMUR, H. 2003. Effects of central and peripheral administration of leptin on pain threshold in rats and mice. *Neuro Endocrinol Lett,* 24**,** 193-6.

KVAM, E., GJERDET, N. R. & BONDEVIK, O. 1987. Traumatic ulcers and pain during orthodontic treatment. *Community dentistry and oral epidemiology,* 15**,** 104-107.

LAC, G., CAVALIE, H., EBAL, E. & MICHAUX, O. 2008. Effects of a high fat diet on bone of growing rats. Correlations between visceral fat, adiponectin and bone mass density. *Lipids in health and disease,* 7**,** 1.

LAKKIS, D., BISSADA, N. F., SABER, A., KHAITAN, L., PALOMO, L., NARENDRAN, S. & AL-ZAHRANI, M. S. 2012. Response to periodontal therapy in patients who had weight loss after bariatric surgery and obese counterparts: a pilot study. *Journal of periodontology,* 83**,** 684-689.

LAMSTER, I. B. & AHLO, J. K. 2007. Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. *Annals of the New York Academy of Sciences,* 1098**,** 216-229.

LAMSTER, I. B., HARTLEY, L. J. & VOGEL, R. I. 1985. Development of a Biochemical Profile for Gingival Crevicular Fluid: Methodological Considerations and Evaluation of Collagen-Degrading and Ground Substance-Degrading Enzyme Activity during Experimental Gingivitis*. *Journal of periodontology,* 56**,** 13-21.

LAROCHELLE, J., FREILER, J., DICE, J. & HAGAN, L. 2007. Plasma resistin levels in asthmatics as a marker of disease state. *Journal of Asthma,* 44**,** 509-513.

LAWRENCE, I. & LIN, K. 1989. A concordance correlation coefficient to evaluate reproducibility. *Biometrics***,** 255-268.

LAZARENKO, O. P., RZONCA, S. O., SUVA, L. J. & LECKA-CZERNIK, B. 2006. Netoglitazone is a PPAR-gamma ligand with selective effects on bone and fat. *Bone,* 38**,** 74-84.

LEAN, M., HAN, T. & SEIDELL, J. 1998. Impairment of health and quality of life in people with large waist circumference. *The Lancet,* 351**,** 853-856.

LEE, K.-J., PARK, Y.-C., YU, H.-S., CHOI, S.-H. & YOO, Y.-J. 2004. Effects of continuous and interrupted orthodontic force on interleukin-1β and prostaglandin E 2 production in gingival crevicular fluid. *American journal of orthodontics and dentofacial orthopedics,* 125**,** 168-177.

LEONARD, M. B., SHULTS, J., WILSON, B. A., TERSHAKOVEC, A. M. & ZEMEL, B. S. 2004. Obesity during childhood and adolescence augments bone mass and bone dimensions. *The American journal of clinical nutrition,* 80**,** 514-523.

LI, Y., HU, B., LIU, Y., DING, G., ZHANG, C. & WANG, S. 2009. The effects of fixed orthodontic appliances on saliva flow rate and saliva electrolyte concentrations. *Journal of oral rehabilitation,* 36**,** 781-785.

LIN, H., MAEDA, K., FUKUHARA, A., SHIMOMURA, I. & ITO, T. 2014. Molecular expression of adiponectin in human saliva. *Biochemical and biophysical research communications,* 445**,** 294-298.

LIN, J., CALLON, K., LIN, C., BAVA, U., ZHENG, M., REID, I. & CORNISH, J. 2007. Alteration of bone cell function by RANKL and OPG in different in vitro models. *European journal of clinical investigation,* 37**,** 407-415.

LITTLE, R. M. 1975. The irregularity index: a quantitative score of mandibular anterior alignment. *American Journal of Orthodontics,* 68**,** 554-563.

LIU, M. & LIU, F. 2014. Regulation of adiponectin multimerization, signaling and function. *Best Practice & Research Clinical Endocrinology & Metabolism,* 28**,** 25-31.

LIU, R. K., CAO, C. F., MENG, H. X. & GAO, Y. 2001. Polymorphonuclear neutrophils and their mediators in gingival tissues from generalized aggressive periodontitis. *Journal of periodontology,* 72**,** 1545-1553.

LIU, Y. E., WANG, M., GREENE, J., SU, J., ULLRICH, S., LI, H., SHENG, S., ALEXANDER, P., SANG, Q. A. & SHI, Y. E. 1997. Preparation and characterization of recombinant tissue inhibitor of metalloproteinase 4 (TIMP-4). *Journal of Biological Chemistry,* 272**,** 20479-20483.

LIU, Z.-W., ZHANG, N., HAN, Q.-Y., ZENG, J.-T., CHU, Y.-L., QIU, J.-M., WANG, Y.-W., MA, L.-T. & WANG, X.-Q. 2005. Correlation of serum leptin levels with anthropometric and metabolic parameters and biochemical liver function in Chinese patients with chronic hepatitis C virus infection. *World journal of gastroenterology,* 11**,** 3357-3362.

LOBSTEIN, T., BAUR, L. & UAUY, R. 2004. Obesity in children and young people: a crisis in public health. *Obesity reviews,* 5**,** 4-85.

LöE, H. & SILNESS, J. 1963. Periodontal disease in pregnancy I. Prevalence and severity. *Acta odontologica scandinavica,* 21**,** 533-551.

LONG, H., WANG, Y., JIAN, F., LIAO, L.-N., YANG, X. & LAI, W.-L. 2016. Current advances in orthodontic pain. *International Journal of Oral Science,* 8**,** 67-75.

LOO, J., YAN, W., RAMACHANDRAN, P. & WONG, D. 2010. Comparative human salivary and plasma proteomes. *Journal of dental research,* 89**,** 1016-1023.

LóPEZ-GóMEZ, J., PEREZ, C. J. & DE LUIS, R. D. 2016. Impact of obesity on bone metabolism. *Endocrinologia y nutricion: organo de la Sociedad Espanola de Endocrinologia y Nutricion*.

LOWNEY, J. J., NORTON, L. A., SHAFER, D. M. & ROSSOMANDO, E. F. 1995. Orthodontic forces increase tumor necrosis factor α in the human gingival sulcus. *American Journal of Orthodontics and Dentofacial Orthopedics,* 108**,** 519-524.

LUCAS, A., QUINLAN, P., ABRAMS, S., RYAN, S., MEAH, S. & LUCAS, P. 1997. Randomised controlled trial of a synthetic triglyceride milk formula for preterm infants. *Archives of Disease in Childhood-Fetal and Neonatal Edition,* 77**,** F178-F184.

LUDWIG, D. S., PETERSON, K. E. & GORTMAKER, S. L. 2001. Relation between consumption of sugar-sweetened drinks and childhood obesity: a prospective, observational analysis. *The Lancet,* 357**,** 505-508.

LUNDIN, M., YUCEL-LINDBERG, T., DAHLLOF, G., MARCUS, C. & MODEER, T. 2004. Correlation between TNFalpha in gingival crevicular fluid and body mass index in obese subjects. *Acta Odontologica Scandinavica,* 62**,** 273-7.

LUPPANAPORNLARP, S., KAJII, T. S., SURARIT, R. & IIDA, J. 2010. Interleukin-1β levels, pain intensity, and tooth movement using two different magnitudes of continuous orthodontic force. *The European Journal of Orthodontics,* 32**,** 596-601.

LYNCH, P., DAVIDOVITCH, Z. & SHANFELD, J. 1988. Interleukin-1β at bone resorption sites: localization during tooth movement in vivo. *Journal of Dental Research,* 67**,** 1474.

MACK, K. B., PHILLIPS, C., JAIN, N. & KOROLUK, L. D. 2013. Relationship between body mass index percentile and skeletal maturation and dental development in orthodontic patients. *American Journal of Orthodontics and Dentofacial Orthopedics,* 143**,** 228-234.

MACLAINE, J., RABIE, A. & WONG, R. 2010. Does orthodontic tooth movement cause an elevation in systemic inflammatory markers? *The European Journal of Orthodontics,* 32**,** 435-440.

MAEDA, N., SHIMOMURA, I., KISHIDA, K., NISHIZAWA, H., MATSUDA, M., NAGARETANI, H., FURUYAMA, N., KONDO, H., TAKAHASHI, M. & ARITA, Y. 2002. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nature medicine,* 8**,** 731-737.

MAH, J. & PRASAD, N. 2004. Dentine phosphoproteins in gingival crevicular fluid during root resorption. *The European Journal of Orthodontics,* 26**,** 25-30.

MAMALI, I., ROUPAS, N. D., ARMENI, A. K., THEODOROPOULOU, A., MARKOU, K. B. & GEORGOPOULOS, N. A. 2012. Measurement of salivary resistin, visfatin and adiponectin levels. *Peptides,* 33**,** 120-124.

MANDEL, I. D. 1990. The diagnostic uses of saliva. *Journal of Oral Pathology & Medicine,* 19**,** 119-125.

MANTIS, N. J., ROL, N. & CORTHESY, B. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal immunology,* 4**,** 603-611.

MäNTYLä, P., STENMAN, M., KINANE, D. F., TIKANOJA, S., LUOTO, H., SALO, T. & SORSA, T. 2003. Gingival crevicular fluid collagenase-2 (MMP-8) test stick for chair-side monitoring of periodontitis. *Journal of periodontal research,* 38**,** 436-439.

MARCACCINI, A. M., AMATO, P. A., LEãO, F. V., GERLACH, R. F. & FERREIRA, J. T. 2010. Myeloperoxidase activity is increased in gingival crevicular fluid and whole saliva after fixed orthodontic appliance activation. *American Journal of Orthodontics and Dentofacial Orthopedics,* 138**,** 613-616.

MARCOVECCHIO, M., GIORGIS, T., DI GIOVANNI, I., CHIAVAROLI, V., CHIARELLI, F. & MOHN, A. 2016. Association between markers of endothelial dysfunction and early signs of renal dysfunction in pediatric obesity and type 1 diabetes. *Pediatric diabetes*.

MASELLA, R. S. & MEISTER, M. 2006. Current concepts in the biology of orthodontic tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 129**,** 458-468.

MATTHEWS, J., ALTMAN, D. G., CAMPBELL, M. & ROYSTON, P. 1990. Analysis of serial measurements in medical research. *Bmj,* 300**,** 230-235.

MAURY, E. & BRICHARD, S. 2010. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Molecular and Cellular Endocrinology,* 314**,** 1-16.

MAVREAS, D. & ATHANASIOU, A. E. 2008. Factors affecting the duration of orthodontic treatment: a systematic review. *The European Journal of Orthodontics,* 30**,** 386-395.

MCGORRAY, S. P., DOLCE, C., KRAMER, S., STEWART, D. & WHEELER, T. T. 2012. A randomized, placebo-controlled clinical trial on the effects of recombinant human relaxin on tooth movement and short-term stability. *American Journal of Orthodontics and Dentofacial Orthopedics,* 141**,** 196-203.

MEGSON, E., FITZSIMMONS, T., DHARMAPATNI, K. & MARK BARTOLD, P. 2010. C-reactive protein in gingival crevicular fluid may be indicative of systemic inflammation. *Journal of clinical periodontology,* 37**,** 797-804.

MEIKLE, M. 2007. Remodeling the dentofacial skeleton: the biological basis of orthodontics and dentofacial orthopedics. *Journal of dental research,* 86**,** 12-24.

MEIKLE, M. C. 2006. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. *The European Journal of Orthodontics,* 28**,** 221-240.

MELSEN, B. 1999. Biological reaction of alveolar bone to orthodontic tooth movement. *Angle Orthodontist,* 69**,** 151-158.

MIKULEWICZ, M. & CHOJNACKA, K. 2010. Trace metal release from orthodontic appliances by in vivo studies: a systematic literature review. *Biological trace element research,* 137**,** 127-138.

MILLER, C. S., KING, C. P., LANGUB, M. C., KRYSCIO, R. J. & THOMAS, M. V. 2006. Salivary biomarkers of existing periodontal disease: a cross-sectional study. *The Journal of the American Dental Association,* 137**,** 322-329.

MIRRIELEES, J., CROFFORD, L. J., LIN, Y., KRYSCIO, R. J., DAWSON III, D. R., EBERSOLE, J. L. & MILLER, C. S. 2010. Rheumatoid arthritis and salivary biomarkers of periodontal disease. *Journal of clinical periodontology,* 37**,** 1068-1074.

MIYAJIMA, K., OHNO, Y., IWATA, T., TANIDA, K. & IIZUKA, T. 1990. The lactic acid and citric acid content in the gingival fluid of orthodontic patients. *Aichi-Gakuin dental science,* 4**,** 75-82.

MIYOSHI, K., IGARASHI, K., SAEKI, S., SHINODA, H. & MITANI, H. 2001. Tooth movement and changes in periodontal tissue in response to orthodontic force in rats vary depending on the time of day the force is applied. *The European Journal of Orthodontics,* 23**,** 329-338.

MODEER, T., BLOMBERG, C., WONDIMU, B., LINDBERG, T. Y. & MARCUS, C. 2011. Association between obesity and periodontal risk indicators in adolescents. *International Journal of Pediatric Obesity,* 6**,** e264-e270.

MODEER, T., BLOMBERG, C. C., WONDIMU, B., JULIHN, A. & MARCUS, C. 2010. Association between obesity, flow rate of whole saliva, and dental caries in adolescents. *Obesity,* 18**,** 2367-2373.

MOGI, M., OTOGOTO, J., OTA, N. & TOGARI, A. 2004. Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with periodontitis. *Journal of Dental Research,* 83**,** 166-169.

MUNRO, C. L., GRAP, M. J., JABLONSKI, R. & BOYLE, A. 2006. Oral health measurement in nursing research: State of the science. *Biological Research for Nursing,* 8**,** 35-42.

MUSSELMAN, D. L., MILLER, A. H., PORTER, M. R., MANATUNGA, A., GAO, F., PENNA, S., PEARCE, B. D., LANDRY, J., GLOVER, S. & MCDANIEL, J. S. 2001. Higher than normal plasma interleukin-6 concentrations in cancer patients

with depression: preliminary findings. *American Journal of Psychiatry,* 158**,** 1252-1257.

MUST, A., PHILLIPS, S. M., TYBOR, D. J., LIVIDINI, K. & HAYES, C. 2012. The association between childhood obesity and tooth eruption. *Obesity,* 20**,** 2070-2074.

MUST, A. & STRAUSS, R. S. 1999. Risks and consequences of childhood and adolescent obesity. *International journal of obesity and related metabolic disorders: Journal of the International Association for the Study of Obesity,* 23**,** S2-11.

NAGASE, H. 1998. Cell surface activation of progelatinase A (proMMP-2) and cell migration. *Cell research,* 8**,** 179-186.

NAGLER, R., SALAMEH, F., REZNICK, A., LIVSHITS, V. & NAHIR, A. 2003. Salivary gland involvement in rheumatoid arthritis and its relationship to induced oxidative stress. *Rheumatology,* 42**,** 1234-1241.

NAHM, D.-S., KIM, H.-J., MAH, J. & BAEK, S.-H. 2004. In vitro expression of matrix metalloproteinase-1, tissue inhibitor of metalloproteinase-1 and transforming growth factor-ß1 in human periodontal ligament fibroblasts. *European Journal of Orthodontics,* 26**,** 129-135.

NAIDOO, T., KONKOL, K., BICCARD, B., DUBOSE, K. & MCKUNE, A. 2012. Elevated salivary C-reactive protein predicted by low cardio-respiratory fitness and being overweight in African children. *Cardiovascular journal of Africa,* 23**,** 501.

NAKAMURA, K., FUSTER, J. J. & WALSH, K. 2014. Adipokines: a link between obesity and cardiovascular disease. *Journal of cardiology,* 63**,** 250-259.

NAKAO, K., GOTO, T., GUNJIGAKE, K., KONOO, T., KOBAYASHI, S. & YAMAGUCHI, K. 2007. Intermittent force induces high RANKL expression in human periodontal ligament cells. *Journal of dental research,* 86**,** 623-628.

NAKASHIMA, T., KOBAYASHI, Y., YAMASAKI, S., KAWAKAMI, A., EGUCHI, K., SASAKI, H. & SAKAI, H. 2000. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-κB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochemical and biophysical research communications,* 275**,** 768-775.

NANDA, R. & KAPILA, S. 2010. Current therapy in Orthodontics. *Orthodontic Journal,* 26**,** 97.

NAVARRO-PALACIOS, A., GARCíA-LóPEZ, E., MEZA-RIOS, A., ARMENDARIZ-BORUNDA, J. & SANDOVAL-RODRíGUEZ, A. 2014. Myeloperoxidase enzymatic activity is increased in patients with different levels of dental crowding after initial orthodontic activation. *American Journal of Orthodontics and Dentofacial Orthopedics,* 146**,** 92-97.

NAVAZESH, M. 1993. Methods for collecting saliva. *Annals of the New York Academy of Sciences,* 694**,** 72-77.

NEELEY II, W. W. & GONZALES, D. A. 2007. Obesity in adolescence: implications in orthodontic treatment. *American Journal of Orthodontics and Dentofacial Orthopedics,* 131**,** 581-588.

NEELEY, W. W. & GONZALES, D. A. 2007. Obesity in adolescence: implications in orthodontic treatment. *American Journal of Orthodontics and Dentofacial Orthopedics,* 131**,** 581-588.

NELSON, S. E., FRANTZ, J. A. & ZIEGLER, E. E. 1998. Absorption of fat and calcium by infants fed a milk-based formula containing palm olein. *Journal of the American College of Nutrition,* 17**,** 327-332.

NEU, M., GOLDSTEIN, M., GAO, D. & LAUDENSLAGER, M. L. 2007. Salivary cortisol in preterm infants: validation of a simple method for collecting saliva for cortisol determination. *Early Human Development,* 83**,** 47-54.

NEWMAN, M. G., TAKEI, H. H., CARRANZA, F. A. & WB, S. 2006. Clinical periodontology. *WB Saunders*.

NG, M., FLEMING, T., ROBINSON, M., THOMSON, B., GRAETZ, N., MARGONO, C., MULLANY, E. C., BIRYUKOV, S., ABBAFATI, C. & ABERA, S. F. 2014. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet,* 384**,** 766-781.

NGAN, P., KESS, B. & WILSON, S. 1989. Perception of discomfort by patients undergoing orthodontic treatment. *American Journal of Orthodontics and Dentofacial Orthopedics,* 96**,** 47-53.

NIBALI, L., D'AIUTO, F., GRIFFITHS, G., PATEL, K., SUVAN, J. & TONETTI, M. S. 2007. Severe periodontitis is associated with systemic inflammation and a dysmetabolic status: a case–control study. *Journal of clinical periodontology,* 34**,** 931-937.

NICHOLLS, S. J., TANG, W. W., BRENNAN, D., BRENNAN, M.-L., MANN, S., NISSEN, S. E. & HAZEN, S. L. 2011. Risk prediction with serial myeloperoxidase monitoring in patients with acute chest pain. *Clinical chemistry,* 57**,** 1762-1770.

NIGRO, E., PIOMBINO, P., SCUDIERO, O., MONACO, M., SCHETTINO, P., CHAMBERY, A. & DANIELE, A. 2015. Evaluation of salivary adiponectin profile in obese patients. *Peptides,* 63**,** 150-155.

NIGRO, E., SCUDIERO, O., MONACO, M. L., PALMIERI, A., MAZZARELLA, G., COSTAGLIOLA, C., BIANCO, A. & DANIELE, A. 2014. New insight into adiponectin role in obesity and obesity-related diseases. *BioMed research international,* 2014.

NIJHUIS, J., RENSEN, S. S., SLAATS, Y., DIELEN, F. M., BUURMAN, W. A. & GREVE, J. W. M. 2009. Neutrophil activation in morbid obesity, chronic activation of acute inflammation. *Obesity,* 17**,** 2014-2018.

NIMERI, G., KAU, C. H., ABOU-KHEIR, N. S. & CORONA, R. 2013. Acceleration of tooth movement during orthodontic treatment-a frontier in orthodontics. *Progress in orthodontics,* 14**,** 1.

NISHIJIMA, Y., YAMAGUCHI, M., KOJIMA, T., AIHARA, N., NAKAJIMA, R. & KASAI, K. 2006. Levels of RANKL and OPG in gingival crevicular fluid during orthodontic tooth movement and effect of compression force on releases from periodontal ligament cells in vitro. *Orthodontics & craniofacial research,* 9**,** 63-70.

NOACK, B., GENCO, R. J., TREVISAN, M., GROSSI, S., ZAMBON, J. J. & NARDIN, E. D. 2001. Periodontal infections contribute to elevated systemic C-reactive protein level. *Journal of periodontology,* 72**,** 1221-1227.

NUMABE, Y., HISANO, A., KAMOI, K., YOSHIE, H., ITO, K. & KURIHARA, H. 2004. Analysis of saliva for periodontal diagnosis and monitoring. *Periodontology,* 40**,** 115-9.

O'CONNOR, P. J. 2000. Patients' perceptions before, during, and after orthodontic treatment. *Journal of clinical orthodontics,* 34**,** 591-592.

OFFENBACHER, S., BARROS, S., MENDOZA, L., MAURIELLO, S., PREISSER, J., MOSS, K., DE JAGER, M. & ASPIRAS, M. 2010. Changes in gingival crevicular fluid inflammatory mediator levels during the induction and resolution of experimental gingivitis in humans. *Journal of clinical periodontology,* 37**,** 324-333.

OGDEN, C. L., CARROLL, M. D., CURTIN, L. R., LAMB, M. M. & FLEGAL, K. M. 2010. Prevalence of high body mass index in US children and adolescents, 2007-2008. *The Journal of the American Medical Association,* 303**,** 242-249.

OGDEN, C. L., CARROLL, M. D., CURTIN, L. R., MCDOWELL, M. A., TABAK, C. J. & FLEGAL, K. M. 2006. Prevalence of overweight and obesity in the United States, 1999-2004. *Journal of the American Medical Association,* 295**,** 1549-1555.

OGDEN, C. L., FLEGAL, K. M., CARROLL, M. D. & JOHNSON, C. L. 2002. Prevalence and trends in overweight among US children and adolescents, 1999-2000. *The Journal of the American Medical Association,* 288**,** 1728-1732.

OKADA, H. & MURAKAMI, S. 1998. Cytokine expression in periodontal health and disease. *Critical Reviews in Oral Biology & Medicine,* 9**,** 248-266.

OKAMOTO, Y., KIHARA, S., FUNAHASHI, T., MATSUZAWA, Y. & LIBBY, P. 2006. Adiponectin: a key adipocytokine in metabolic syndrome. *Clinical Science,* 110**,** 267-278.

OKESON, J. P. 2005. *Bell's orofacial pains: the clinical management of orofacial pain,* Quintessence Publishing Company Chicago, Ill, USA.

OSHIMA, K., NAMPEI, A., MATSUDA, M., IWAKI, M., FUKUHARA, A., HASHIMOTO, J., YOSHIKAWA, H. & SHIMOMURA, I. 2005. Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast. *Biochemical and biophysical research communications,* 331**,** 520-526.

OSHIRO, T., SHIOTANI, A., SHIBASAKI, Y. & SASAKI, T. 2002. Osteoclast induction in periodontal tissue during experimental movement of incisors in osteoprotegerin-deficient mice. *The Anatomical Record,* 266**,** 218-225.

OSTBERG, A. L., BENGTSSON, C., LISSNER, L. & HAKEBERG, M. 2012. Oral health and obesity indicators. *BMC Oral Health,* 12**,** 50.

OTASEVIC, M., NAINI, F. B., GILL, D. S. & LEE, R. T. 2006. Prospective randomized clinical trial comparing the effects of a masticatory bite wafer and avoidance of hard food on pain associated with initial orthodontic tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 130**,** 6. e9-6. e15.

OTERO, L., GARCíA, D. A. & WILCHES-BUITRAGO, L. 2016. Expression and Presence of OPG and RANKL mRNA and Protein in Human Periodontal Ligament with Orthodontic Force. *Gene regulation and systems biology,* 10**,** 15.

OUCHI, N., KIHARA, S., ARITA, Y., MAEDA, K., KURIYAMA, H., OKAMOTO, Y., HOTTA, K., NISHIDA, M., TAKAHASHI, M. & NAKAMURA, T. 1999. Novel modulator for endothelial adhesion molecules adipocyte-derived plasma protein adiponectin. *Circulation,* 100**,** 2473-2476.

OUCHI, N., KIHARA, S., FUNAHASHI, T., MATSUZAWA, Y. & WALSH, K. 2003. Obesity, adiponectin and vascular inflammatory disease. *Current opinion in lipidology,* 14**,** 561-566.

OUCHI, N., KOBAYASHI, H., KIHARA, S., KUMADA, M., SATO, K., INOUE, T., FUNAHASHI, T. & WALSH, K. 2004. Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *Journal of Biological Chemistry,* 279**,** 1304-1309.

OUCHI, N., PARKER, J. L., LUGUS, J. J. & WALSH, K. 2011. Adipokines in inflammation and metabolic disease. *Nature Reviews Immunology,* 11**,** 85-97.

ÖZCAN, E., SAYGUN, N. I., SERDAR, M. A. & KURT, N. 2015. Evaluation of the salivary levels of visfatin, chemerin, and progranulin in periodontal inflammation. *Clinical oral investigations,* 19**,** 921-928.

PALMER, A. K. & KIRKLAND, J. L. 2016. Aging and adipose tissue: potential interventions for diabetes and regenerative medicine. *Experimental gerontology*.

PANDIS, N., POLYCHRONOPOULOU, A. & ELIADES, T. 2007. Self-ligating vs conventional brackets in the treatment of mandibular crowding: a prospective clinical trial of treatment duration and dental effects. *American Journal of Orthodontics and Dentofacial Orthopedics,* 132**,** 208-215.

PANNUNZIO, E., AMANCIO, O. M. S., VITALLE, M. S. D. S., SOUZA, D. N. D., MENDES, F. M. & NICOLAU, J. 2010. Analysis of the stimulated whole saliva in overweight and obese school children. *Revista da Associação Médica Brasileira,* 56**,** 32-36.

PAPAGEORGIOU, S., KONSTANTINIDIS, I., PAPADOPOULOU, K., JäGER, A. & BOURAUEL, C. 2014a. A systematic review and meta-analysis of experimental clinical evidence on initial aligning archwires and archwire sequences. *Orthodontics & craniofacial research,* 17**,** 197-215.

PAPAGEORGIOU, S. N., GöLZ, L., JäGER, A., ELIADES, T. & BOURAUEL, C. 2016. Lingual vs. labial fixed orthodontic appliances: systematic review and meta-analysis of treatment effects. *European journal of oral sciences*.

PAPAGEORGIOU, S. N., KONSTANTINIDIS, I., PAPADOPOULOU, K., JäGER, A. & BOURAUEL, C. 2014b. Clinical effects of pre-adjusted edgewise orthodontic brackets: a systematic review and meta-analysis. *The European Journal of Orthodontics,* 36**,** 350-363.

PAPAGEORGIOU, S. N., REICHERT, C., JäGER, A. & DESCHNER, J. 2015a. Effect of overweight/obesity on response to periodontal treatment: systematic review and a meta-analysis. *Journal of clinical periodontology,* 42**,** 247-261.

PAPAGEORGIOU, S. N., XAVIER, G. M. & COBOURNE, M. T. 2015b. Basic study design influences the results of orthodontic clinical investigations. *Journal of clinical epidemiology,* 68**,** 1512-1522.

PARASKEVAS, S., HUIZINGA, J. D. & LOOS, B. G. 2008. A systematic review and meta-analyses on C-reactive protein in relation to periodontitis. *Journal of clinical periodontology,* 35**,** 277-290.

PARK, H. K. & AHIMA, R. S. 2013. Resistin in rodents and humans. *Diabetes & metabolism journal,* 37**,** 404-414.

PASCHOS, E., LIMBACH, M., TEICHMANN, M., HUTH, K. C., FOLWACZNY, M., HICKEL, R. & RUDZKI-JANSON, I. 2008. Orthodontic attachments and chlorhexidine-containing varnish effects on gingival health. *The Angle Orthodontist,* 78**,** 908-916.

PASQUIN, S., SHARMA, M. & GAUCHAT, J.-F. 2016. Cytokines of the LIF/CNTF family and metabolism. *Cytokine,* 82**,** 122-124.

PASSARELLA, S., DE BARI, L., VALENTI, D., PIZZUTO, R., PAVENTI, G. & ATLANTE, A. 2008. Mitochondria and L-lactate metabolism. *FEBS letters,* 582**,** 3569-3576.

PATEL, L., BUCKELS, A. C., KINGHORN, I. J., MURDOCK, P. R., HOLBROOK, J. D., PLUMPTON, C., MACPHEE, C. H. & SMITH, S. A. 2003. Resistin is expressed in human macrophages and directly regulated by PPARγ activators. *Biochemical and biophysical research communications,* 300**,** 472-476.

PATEL, S., MCGORRAY, S. P., YEZIERSKI, R., FILLINGIM, R., LOGAN, H. & WHEELER, T. T. 2011. Effects of analgesics on orthodontic pain. *American Journal of Orthodontics and Dentofacial Orthopedics,* 139**,** e53-e58.

PATEL, S. P. & RAJU, P. A. 2014. Gingival crevicular fluid and serum levels of resistin in obese and non-obese subjects with and without periodontitis and association with single nucleotide polymorphism at− 420. *Journal of Indian Society of Periodontology,* 18**,** 555.

PATEL, V. 1989. *Non-completion of Orthodontic Treatment: A Study of Patient and Parent Factors Contributing to Discontinuation in the Hospital Service and a Specialist Practice*, V. Patel.

PEDERSON, E., STANKE, S., WHITENER, S., SEBASTIANI, P., LAMBERTS, B. & TURNER, D. 1995. Salivary levels of α 2-macroglobulin, α 1-antitrypsin, C-reactive protein, cathepsin G and elastase in humans with or without destructive periodontal disease. *Archives of oral biology,* 40**,** 1151-1155.

PENDER, N., SAMUELS, R. & LAST, K. 1994. The monitoring of orthodontic tooth movement over a 2-year period by analysis of gingival crevicular fluid. *The European Journal of Orthodontics,* 16**,** 511-520.

PERINETTI, G., PAOLANTONIO, M., D'ATTILIO, M., D'ARCHIVIO, D., TRIPODI, D., FEMMINELLA, B., FESTA, F. & SPOTO, G. 2002. Alkaline phosphatase activity in gingival crevicular fluid during human orthodontic tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 122**,** 548-556.

PERINETTI, G., PRIMOŽIČ, J., CASTALDO, A., DI LENARDA, R. & CONTARDO, L. 2013. Is gingival crevicular fluid volume sensitive to orthodontic tooth movement? A systematic review of split-mouth longitudinal studies. *Orthodontics & craniofacial research,* 16**,** 1-19.

PERLSTEIN, M. I. & BISSADA, N. F. 1977. Influence of obesity and hypertension on the severity of periodontitis in rats. *Oral Surgery, Oral Medicine, Oral Pathology,* 43**,** 707-719.

PEROS, K., MESTROVIC, S., ANIC-MILOSEVIC, S. & SLAJ, M. 2011. Salivary microbial and nonmicrobial parameters in children with fixed orthodontic appliances. *The Angle Orthodontist,* 81**,** 901-906.

PFAFFE, T., COOPER-WHITE, J., BEYERLEIN, P., KOSTNER, K. & PUNYADEERA, C. 2011. Diagnostic potential of saliva: current state and future applications. *Clinical chemistry,* 57**,** 675-687.

PFEILSCHIFTER, J., KÖDITZ, R., PFOHL, M. & SCHATZ, H. 2002. Changes in proinflammatory cytokine activity after menopause. *Endocrine reviews,* 23**,** 90-119.

PHALIPON, A., CARDONA, A., KRAEHENBUHL, J.-P., EDELMAN, L., SANSONETTI, P. J. & CORTHESY, B. 2002. Secretory component: a new role in secretory IgA-mediated immune exclusion in vivo. *Immunity,* 17**,** 107-115.

PIERPONT, Y. N., DINH, T. P., SALAS, R. E., JOHNSON, E. L., WRIGHT, T. G., ROBSON, M. C. & PAYNE, W. G. 2014. Obesity and surgical wound healing: a current review. *International Scholarly Research Network Obesity,* 2014.

PIESTRZENIEWICZ, K., ŁUCZAK, K., KOMOROWSKI, J., MACIEJEWSKI, M., WIKA, J. J. & GOCH, J. H. 2008. Resistin increases with obesity and atherosclerotic risk factors in patients with myocardial infarction. *Metabolism,* 57**,** 488-493.

PIETROBELLI, A., KERNS, J. & FAITH, M. 2005. Childhood obesity: Global trends, health complications, and prevention needs. *World Journal of Pediatrics,* 1.

PILCHER, B. K., WANG, M., QIN, X. J., PARKS, W. C., SENIOR, R. M. & WELGUS, H. G. 1999. Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. *Annals of the New York Academy of Sciences,* 878**,** 12-24.

PILON, J. J., KUIJPERS-JAGTMAN, A. M. & MALTHA, J. C. 1996. Magnitude of orthodontic forces and rate of bodily tooth movement. An experimental study. *American Journal of Orthodontics and Dentofacial Orthopedics,* 110**,** 16-23.

PINKA, R., SIMEKA, J., VONDRAKOVAB, J., FABERB, E., MICHLA, P., PAZDERAA, J. & INDRAKB, K. 2009. Saliva as a diagnostic medium. *Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia* 153**,** 103-110.

POLAT, O., KARAMAN, A. I. & DURMUS, E. 2005. Effects of preoperative ibuprofen and naproxen sodium on orthodontic pain. *The Angle orthodontist,* 75**,** 791-796.

POWERS, P. S., HOLLAND, P., MILLER, C. & POWERS, H. P. 1981. Salivation patterns of obese and normal subjects. *International journal of obesity,* 6**,** 267-270.

PRADEEP, A., PRIYANKA, N., PRASAD, M., KALRA, N. & KUMARI, M. 2012. Association of progranulin and high sensitivity CRP concentrations in gingival crevicular fluid and serum in chronic periodontitis subjects with and without obesity. *Disease markers,* 33**,** 207-213.

PRICE, R., ASENJO, J., CHRISTOU, N., BACKMAN, S. & SCHWEINHARDT, P. 2013. The role of excess subcutaneous fat in pain and sensory sensitivity in obesity. *European Journal of Pain,* 17**,** 1316-1326.

PRINGLE, A. M., PETRIE, A., CUNNINGHAM, S. J. & MCKNIGHT, M. 2009. Prospective randomized clinical trial to compare pain levels associated with 2 orthodontic fixed bracket systems. *American Journal of Orthodontics and Dentofacial Orthopedics,* 136**,** 160-167.

PRLIC, M. & BEVAN, M. J. 2006. An antibody paradox, resolved. *Science,* 311**,** 1875-1876.

PROCACCINI, C., JIRILLO, E. & MATARESE, G. 2012. Leptin as an immunomodulator. *Molecular aspects of medicine,* 33**,** 35-45.

PROCTOR, G. B. & CARPENTER, G. H. 2007. Regulation of salivary gland function by autonomic nerves. *Autonomic Neuroscience,* 133**,** 3-18.

PROFFIT, W. R., FIELDS JR, H. W. & SARVER, D. M. 2014. *Contemporary orthodontics,* Elsevier Health Sciences.

PULFORD, D. J., MOSTELLER, M., BRILEY, J. D., JOHANSSON, K. W. & NELSEN, A. J. 2013. Saliva sampling in global clinical studies: the impact of low sampling volume on performance of DNA in downstream genotyping experiments. *BMC medical genomics,* 6**,** 20.

QI, Q., CHU, A. Y., KANG, J. H., HUANG, J., ROSE, L. M., JENSEN, M. K., LIANG, L., CURHAN, G. C., PASQUALE, L. R., WIGGS, J. L., DE VIVO, I., CHAN, A. T., CHOI, H. K., TAMIMI, R. M., RIDKER, P. M., HUNTER, D. J., WILLETT, W. C., RIMM, E. B., CHASMAN, D. I., HU, F. B. & QI, L. 2014. Fried food consumption, genetic risk, and body mass index: gene-diet interaction analysis in three US cohort studies. *BMJ,* 348**,** g1610.

QUASNICHKA, H. L., ANDERSON-MACKENZIE, J. M., TARLTON, J. F., SIMS, T. J., BILLINGHAM, M. E. & BAILEY, A. J. 2005. Cruciate ligament laxity and femoral intercondylar notch narrowing in early-stage knee osteoarthritis. *Arthritis & Rheumatism,* 52**,** 3100-3109.

RAFFETTO, J. D. & KHALIL, R. A. 2008. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. *Biochemical pharmacology,* 75**,** 346-359.

RAHMAN, S., SPENCER, R. J., LITTLEWOOD, S. J., O'DYWER, L., BARBER, S. K. & RUSSELL, J. S. 2015. A multicenter randomized controlled trial to compare a self-

ligating bracket with a conventional bracket in a UK population: Part 2: Pain perception. *The Angle Orthodontist,* 86**,** 149-156.

RAMACHANDRAN, A., SNEHALATHA, C., VINITHA, R., THAYYIL, M., SATHISH KUMAR, C., SHEEBA, L., JOSEPH, S. & VIJAY, V. 2002. Prevalence of overweight in urban Indian adolescent school children. *Diabetes research and clinical practice,* 57**,** 185-190.

RAMFJORD, S. P. 1959. Indices for prevalence and incidence of periodontal disease. *Journal of periodontology,* 30**,** 51-59.

RAMSEIER, C. A., KINNEY, J. S., HERR, A. E., BRAUN, T., SUGAI, J. V., SHELBURNE, C. A., RAYBURN, L. A., TRAN, H. M., SINGH, A. K. & GIANNOBILE, W. V. 2009. Identification of pathogen and host-response markers correlated with periodontal disease. *Journal of Periodontology,* 80**,** 436-446.

RANDEVA, H. S., KARTERIS, E., LEWANDOWSKI, K. C., SAILESH, S., O'HARE, P. & HILLHOUSE, E. W. 2003. Circadian rhythmicity of salivary leptin in healthy subjects. *Molecular genetics and metabolism,* 78**,** 229-235.

RANGE, H., POITOU, C., BOILLOT, A., CIANGURA, C., KATSAHIAN, S., LACORTE, J.-M., CZERNICHOW, S., MEILHAC, O., BOUCHARD, P. & CHAUSSAIN, C. 2013. Orosomucoid, a new biomarker in the association between obesity and periodontitis. *PloS one,* 8**,** e57645.

RAUTEMAA, R., JäRVENSIVU, A., KARI, K., WAHLGREN, J., DECARLO, A., RICHARDSON, M. & SORSA, T. 2004. Intracellular localization of Porphyromonas gingivalis thiol proteinase in periodontal tissues of chronic periodontitis patients. *Oral diseases,* 10**,** 298-305.

RAVN, P., CIZZA, G., BJARNASON, N., THOMPSON, D., DALEY, M., WASNICH, R., MCCLUNG, M., HOSKING, D., YATES, A. & CHRISTIANSEN, C. 1999. Low body mass index is an important risk factor for low bone mass and increased bone loss in early postmenopausal women. *Journal of bone and mineral research,* 14**,** 1622-1627.

REDLICH, M., RAHAMIM, E., GAFT, A. & SHOSHAN, S. 1996. The response of supraalveolar gingival collagen to orthodontic rotation movement in dogs. *American journal of orthodontics and dentofacial orthopedics,* 110**,** 247-255.

REDLICH, M., REICHENBERG, E., HARARI, D., ZAKS, B., SHOSHAN, S. & PALMON, A. 2001. The effect of mechanical force on mRNA levels of collagenase, collagen type I, and tissue inhibitors of metalloproteinases in gingivae of dogs. *Journal of dental research,* 80**,** 2080-2084.

REID, I. R. 2002. Relationships among body mass, its components, and bone. *BoneKEy-Osteovision.*

REILLY, J. J., ARMSTRONG, J., DOROSTY, A. R., EMMETT, P. M., NESS, A., ROGERS, I., STEER, C., SHERRIFF, A., AVON LONGITUDINAL STUDY OF, P. & CHILDREN STUDY, T. 2005. Early life risk factors for obesity in childhood: cohort study. *BMJ,* 330**,** 1357.

REITAN, K. 1956. Selecting forces in orthodontics. *European Orthodontic Society Transactions,* 32**,** 108-126.

REITAN, K. 1960. Tissue behavior during orthodontic tooth movement. *American Journal of Orthodontics,* 46**,** 881-900.

REN, Y., MALTHA, J. C., STOKROOS, I., LIEM, R. S. & KUIJPERS-JAGTMAN, A. M. 2008. Effect of duration of force application on blood vessels in young and adult rats. *American Journal of Orthodontics and Dentofacial Orthopedics,* 133**,** 752-757.

REN, Y., MALTHA, J. C., VON DEN HOFF, J. W., KUIJPERS-JAGTMAN, A. M. & ZHANG, D. 2002. Cytokine levels in crevicular fluid are less responsive to orthodontic force in adults than in juveniles. *Journal of clinical periodontology,* 29**,** 757-762.

RHIE, Y. J., LEE, K. H., CHUNG, S. C., KIM, H. S. & KIM, D. H. 2010. Effects of body composition, leptin, and adiponectin on bone mineral density in prepubertal girls. *Journal of Korean medical science,* 25**,** 1187-1190.

RICCI, R. & BEVILACQUA, F. 2012. The potential role of leptin and adiponectin in obesity: a comparative review. *The Veterinary Journal,* 191**,** 292-298.

RIDKER, P. M. 2003. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. *Circulation,* 107**,** 363-369.

RIDKER, P. M. & SILVERTOWN, J. D. 2008. Inflammation, C-reactive protein, and atherothrombosis. *Journal of periodontology,* 79**,** 1544-1551.

RILEY, M. & BEARN, D. R. 2009. A systematic review of clinical trials of aligning archwires. *Journal of Orthodontics,* 36**,** 42-51.

RITCHIE, C. S. 2007. Obesity and periodontal disease. *Periodontology 2000,* 44**,** 154-163.

ROBINSON, T. N. 2001. Television viewing and childhood obesity. *Pediatric Clinics of North America,* 48**,** 1017-1025.

ROCHE, A. F., HEYMSFIELD, S. B. & LOHMAN, T. G. 1996. *Human body composition,* Human Kinetics Publishers.

RODY JR, W. J., AKHLAGHI, H., AKYALCIN, S., WILTSHIRE, W. A., WIJEGUNASINGHE, M. & FILHO, G. N. 2011. Impact of orthodontic retainers on periodontal health status assessed by biomarkers in gingival crevicular fluid. *The Angle Orthodontist,* 81**,** 1083-1089.

RODY, W. J., KING, G. J. & GU, G. 2001. Osteoclast recruitment to sites of compression in orthodontic tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 120**,** 477-489.

ROSC, D., ADAMCZYK, P., BOINSKA, J., SZAFKOWSKI, R., PONIKOWSKA, I., STANKOWSKA, K., GORALCZYK, B. & RUSZKOWSKA-CIASTEK, B. 2015. CRP, but not TNF-alpha or IL-6, decreases after weight loss in patients with morbid obesity exposed to intensive weight reduction and balneological treatment. *Journal of Zhejiang University-SCIENCE B,* 16**,** 404-11.

ROSEN, E. D. & SPIEGELMAN, B. M. 2006. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature,* 444**,** 847-853.

ROSSOMANDO, E., KENNEDY, J. & HADJIMICHAEL, J. 1990. Tumour necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. *Archives of Oral Biology,* 35**,** 431-434.

ROSVALL, M. D., FIELDS, H. W., ZIUCHKOVSKI, J., ROSENSTIEL, S. F. & JOHNSTON, W. M. 2009. Attractiveness, acceptability, and value of orthodontic appliances. *American Journal of Orthodontics and Dentofacial Orthopedics,* 135**,** 276. e1-276. e12.

RUDOLPH, V., GOLDMANN, B. U., BöS, C., RUDOLPH, T. K., KLINKE, A., FRIEDRICHS, K., LAU, D., WEGSCHEIDER, K., HADDAD, M. & MEINERTZ, T. 2011. Diagnostic value of MPO plasma levels in patients admitted for suspected myocardial infarction. *International journal of cardiology,* 153**,** 267-271.

RUDOLPH, V., RUDOLPH, T. K., HENNINGS, J. C., BLANKENBERG, S., SCHNABEL, R., STEVEN, D., HADDAD, M., KNITTEL, K., WENDE, S. & WENZEL, J. 2007. Activation of polymorphonuclear neutrophils in patients with

impaired left ventricular function. *Free Radical Biology and Medicine,* 43**,** 1189-1196.

RUDRAKSHI, C., SRINIVAS, N. & MEHTA, D. 2011. A comparative evaluation of hepatocyte growth factor levels in gingival crevicular fluid and saliva and its correlation with clinical parameters in patients with and without chronic periodontitis: A clinico-biochemical study. *Journal of Indian Society of Periodontology,* 15**,** 147.

RYGH, P. 1973. Ultrastructural changes in pressure zones of human periodontium incident to orthodontic tooth movement. *Acta Odontologica Scandinavica,* 31**,** 109-122.

RYGH, P., BOWLING, K., HOVLANDSDAL, L. & WILLIAMS, S. 1986. Activation of the vascular system: a main mediator of periodontal fiber remodeling in orthodontic tooth movement. *American journal of orthodontics,* 89**,** 453-468.

RYGH, P. & BRUDVIK, P. 1995. The histological responses of the periodontal ligament to horizontal orthodontic loads. *The periodontal ligament in health and disease. St Louis: Mosby*.

RYO, M., NAKAMURA, T., KIHARA, S., KUMADA, M., SHIBAZAKI, S., TAKAHASHI, M., NAGAI, M., MATSUZAWA, Y. & FUNAHASHI, T. 2004. Adiponectin as a biomarker of the metabolic syndrome. *Circulation Journal,* 68**,** 975-981.

SACERDOTE, P. & LEVRINI, L. 2012. Peripheral mechanisms of dental pain: the role of substance P. *Mediators of inflammation,* 2012.

SADEGHIANRIZI, A., FORSBERG, C.-M., MARCUS, C. & DAHLLöF, G. 2005. Craniofacial development in obese adolescents. *The European Journal of Orthodontics,* 27**,** 550-555.

SAITO, T., YAMAGUCHI, N., SHIMAZAKI, Y., HAYASHIDA, H., YONEMOTO, K., DOI, Y., KIYOHARA, Y., IIDA, M. & YAMASHITA, Y. 2008. Serum levels of resistin and adiponectin in women with periodontitis: the Hisayama study. *Journal of Dental Research,* 87**,** 319-322.

SAKELLARI, D., MENTI, S. & KONSTANTINIDIS, A. 2008. Free soluble receptor activator of nuclear factor-κb ligand in gingival crevicular fluid correlates with distinct pathogens in periodontitis patients. *Journal of clinical periodontology,* 35**,** 938-943.

SALAMAT, M. R., SALAMAT, A. H. & JANGHORBANI, M. 2016. Association between Obesity and Bone Mineral Density by Gender and Menopausal Status. *Endocrinology and Metabolism,* 31.

SALLA, J. T., DE ALBUQUERQUE TADDEI, S. R., QUEIROZ-JUNIOR, C. M., JUNIOR, I. A., TEIXEIRA, M. M. & SILVA, T. A. 2012. The effect of IL-1 receptor antagonist on orthodontic tooth movement in mice. *Archives of Oral Biology,* 57**,** 519-524.

SALONEN, E.-M., VARTIO, T., HEDMAN, K. & VAHERI, A. 1984. Binding of fibronectin by the acute phase reactant C-reactive protein. *Journal of Biological Chemistry,* 259**,** 1496-1501.

SAMUELS, R., RUDGE, S. & MAIR, L. 1998. A clinical study of space closure with nickel-titanium closed coil springs and an elastic module. *American Journal of Orthodontics and Dentofacial Orthopedics,* 114**,** 73-79.

SANDHU, S. S., CHEEMA, M. S. & KHEHRA, H. S. 2016. Comparative effectiveness of pharmacologic and nonpharmacologic interventions for orthodontic pain relief at peak pain intensity: A Bayesian network meta-analysis. *American Journal of Orthodontics and Dentofacial Orthopedics,* 150**,** 13-32.

SANDY, J. 1992. Tooth eruption and orthodontic movement. *British dental journal,* 172**,** 141-149.

SANDY, J. R., FARNDALE, R. W. & MEIKLE, M. C. 1993. Recent advances in understanding mechanically induced bone remodeling and their relevance to orthodontic theory and practice. *American Journal of Orthodontics and Dentofacial Orthopedics,* 103**,** 212-222.

SARI, E. & UCAR, C. 2007. Interleukin 1β levels around microscrew implants during orthodontic tooth movement. *The Angle orthodontist,* 77**,** 1073-1078.

SARLATI, F., AKHONDI, N., ETTEHAD, T., NEYESTANI, T. & KAMALI, Z. 2008. Relationship between obesity and periodontal status in a sample of young Iranian adults. *International Dental Journal,* 58**,** 36-40.

SCHAPHER, M., WENDLER, O., GRöSCHL, M., SCHäFER, R., IRO, H. & ZENK, J. 2009. Salivary leptin as a candidate diagnostic marker in salivary gland tumors. *Clinical chemistry,* 55**,** 914-922.

SCHERER, P. E., WILLIAMS, S., FOGLIANO, M., BALDINI, G. & LODISH, H. F. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. *Journal of Biological chemistry,* 270**,** 26746-26749.

SCHEURER, P. A., FIRESTONE, A. R. & BURGIN, W. B. 1996. Perception of pain as a result of orthodontic treatment with fixed appliances. *The European Journal of Orthodontics,* 18**,** 349-357.

SCHIPPER, R. G., SILLETTI, E. & VINGERHOEDS, M. H. 2007. Saliva as research material: biochemical, physicochemical and practical aspects. *Archives of oral biology,* 52**,** 1114-1135.

SCHOPPET, M., PREISSNER, K. T. & HOFBAUER, L. C. 2002. RANK ligand and osteoprotegerin paracrine regulators of bone metabolism and vascular function. *Arteriosclerosis, thrombosis, and vascular biology,* 22**,** 549-553.

SCHOTT, T. C. & LUDWIG, B. 2014. Quantification of wear-time adherence of removable appliances in young orthodontic patients in relation to their BMI: a preliminary study. *Patient Prefer Adherence,* 8**,** 1587-1595.

SCHULZ, K. F. & GRIMES, D. A. 2005. Sample size calculations in randomised trials: mandatory and mystical. *The Lancet,* 365**,** 1348-1353.

SCOTT, P., DIBIASE, A. T., SHERRIFF, M. & COBOURNE, M. T. 2008a. Alignment efficiency of Damon3 self-ligating and conventional orthodontic bracket systems: a randomized clinical trial. *American Journal of Orthodontics and Dentofacial Orthopedics,* 134**,** 470. e1-470. e8.

SCOTT, P., SHERRIFF, M., DIBIASE, A. T. & COBOURNE, M. T. 2008b. Perception of discomfort during initial orthodontic tooth alignment using a self-ligating or conventional bracket system: a randomized clinical trial. *The European Journal of Orthodontics,* 30**,** 227-232.

SEGAL, G., SCHIFFMAN, P. & TUNCAY, O. 2004. Meta analysis of the treatment-related factors of external apical root resorption. *Orthodontics & craniofacial research,* 7**,** 71-78.

SEN, B., XIE, Z., CASE, N., MA, M., RUBIN, C. & RUBIN, J. 2008. Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable β-catenin signal. *Endocrinology,* 149**,** 6065-6075.

SEO, B.-M., MIURA, M., GRONTHOS, S., MARK BARTOLD, P., BATOULI, S., BRAHIM, J., YOUNG, M., GEHRON ROBEY, P., WANG, C. Y. & SHI, S. 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. *The Lancet,* 364**,** 149-155.

SERGL, H. G., KLAGES, U. & ZENTNER, A. 1998. Pain and discomfort during orthodontic treatment: causative factors and effects on compliance. *American Journal of Orthodontics and Dentofacial Orthopedics,* 114**,** 684-691.

SERGL, H. G., KLAGES, U. & ZENTNER, A. 2000. Functional and social discomfort during orthodontic treatment-effects on compliance and prediction of patients' adaptation by personality variables. *The European Journal of Orthodontics,* 22**,** 307-315.

SEXTON, W. M., LIN, Y., KRYSCIO, R. J., DAWSON, D. R., EBERSOLE, J. L. & MILLER, C. S. 2011. Salivary biomarkers of periodontal disease in response to treatment. *Journal of clinical periodontology,* 38**,** 434-441.

SEYMOUR, R. 1982. The use of pain scales in assessing the efficacy of analgesics in post-operative dental pain. *European journal of Clinical Pharmacology,* 23**,** 441-444.

SHAPIRO, L. & SCHERER, P. E. 1998. The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Current Biology,* 8**,** 335-340.

SHIMADA, Y., KOMATSU, Y., IKEZAWA-SUZUKI, I., TAI, H., SUGITA, N. & YOSHIE, H. 2010. The effect of periodontal treatment on serum leptin, interleukin-6, and C-reactive protein. *Journal of periodontology,* 81**,** 1118-1123.

SHIR, Y., ZELTSER, R., VATINE, J. J., CARMI, G., BELFER, I., ZANGEN, A., OVERSTREET, D., RABER, P. & SELTZER, Z. 2001. Correlation of intact sensibility and neuropathic pain-related behaviors in eight inbred and outbred rat strains and selection lines. *Pain,* 90**,** 75-82.

SHOWKATBAKHSH, R., JAMILIAN, A. & SHOWKATBAKHSH, M. 2010. The effect of pulsed electromagnetic fields on the acceleration of tooth movement. *World J Orthod,* 11**,** e52-e56.

SIBRAA, P. D., REINHARDT, R., DYER, J. & DUBOIS, L. 1991. Acute-phase protein detection and quantification in gingival crevicular fluid by direct and indirect immunodot. *Journal of clinical periodontology,* 18**,** 101-106.

SIERRA-HONIGMANN, M. R. O., NATH, A. K., MURAKAMI, C., GARCíA-CARDEñA, G., PAPAPETROPOULOS, A., SESSA, W. C., MADGE, L. A., SCHECHNER, J. S., SCHWABB, M. B. & POLVERINI, P. J. 1998. Biological action of leptin as an angiogenic factor. *Science,* 281**,** 1683-1686.

SILNESS, J. & LöE, H. 1964. Periodontal disease in pregnancy II. Correlation between oral hygiene and periodontal condition. *Acta odontologica scandinavica,* 22**,** 121-135.

SILSWAL, N., SINGH, A. K., ARUNA, B., MUKHOPADHYAY, S., GHOSH, S. & EHTESHAM, N. Z. 2005. Human resistin stimulates the pro-inflammatory cytokines TNF-α and IL-12 in macrophages by NF-κB-dependent pathway. *Biochemical and biophysical research communications,* 334**,** 1092-1101.

SILVER, J. G., MARTIN, A. W. & MCBRIDE, B. C. 1977. Experimental transient bacteraemias in human subjects with varying degrees of plaque accumulation and gingival inflammation. *Journal of clinical periodontology,* 4**,** 92-99.

SKöLD-LARSSON, K., YUCEL-LINDBERG, T., TWETMAN, S. & MODEER, T. 2003. Effect of a triclosan-containing dental gel on the levels of prostaglandin I2 and interleukin-1 β in gingival crevicular fluid from adolescents with fixed orthodontic appliances. *Acta Odontologica,* 61**,** 193-196.

SLADE, G., OFFENBACHER, S., BECK, J., HEISS, G. & PANKOW, J. 2000. Acute-phase inflammatory response to periodontal disease in the US population. *Journal of Dental Research,* 79**,** 49-57.

SŁOTWIŃSKA, S. M. & SŁOTWIŃSKI, R. 2015. Host response, obesity, and oral health. *Central-European Journal of Immunology,* 40**,** 201.

SMITH, S., SUMAR, B. & DIXON, K. 2014. Musculoskeletal pain in overweight and obese children. *International Journal of Obesity,* 38**,** 11-15.

SNOEK, P. & VON DEN HOFF, J. W. 2005. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors.

SOBUE, T., HAKEDA, Y., KOBAYASHI, Y., HAYAKAWA, H., YAMASHITA, K., AOKI, T., KUMEGAWA, M., NOGUCHI, T. & HAYAKAWA, T. 2001. Tissue inhibitor of metalloproteinases 1 and 2 directly stimulate the bone-resorbing activity of isolated mature osteoclasts. *Journal of Bone and Mineral Research,* 16**,** 2205-2214.

SOCRANSKY, S., HAFFAJEE, A., GOODSON, J. & LINDHE, J. 1984. New concepts of destructive periodontal disease. *Journal of clinical periodontology,* 11**,** 21-32.

SONESSON, M., ERICSON, D., KINNBY, B. & WICKSTRöM, C. 2011. Glycoprotein 340 and sialic acid in minor-gland and whole saliva of children, adolescents, and adults. *European journal of oral sciences,* 119**,** 435-440.

SOUKUP, M., BIESIADA, I., HENDERSON, A., IDOWU, B., RODEBACK, D., RIDPATH, L., BRIDGES, E. G., NAZAR, A. M. & BRIDGES, K. G. 2012. Salivary uric acid as a noninvasive biomarker of metabolic syndrome. *Diabetology & metabolic syndrome,* 4**,** 1.

SOUZA-COSTA, D. C., SANDRIM, V. C., LOPES, L. F., GERLACH, R. F., REGO, E. M. & TANUS-SANTOS, J. E. 2007. Anti-inflammatory effects of atorvastatin: modulation by the T-786C polymorphism in the endothelial nitric oxide synthase gene. *Atherosclerosis,* 193**,** 438-444.

SPROGAR, Š., VAUPOTIC, T., CöR, A., DREVENŠEK, M. & DREVENŠEK, G. 2008. The endothelin system mediates bone modeling in the late stage of orthodontic tooth movement in rats. *Bone,* 43**,** 740-747.

SRIKANTH BABU, B. M., PULLA REDDY, B., PRIYA, V. H. S., MUNSHI, A., SUREKHA RANI, H., SUMAN LATHA, G., RAO, V. D. & JYOTHY, A. 2012. Cytokine gene polymorphisms in the susceptibility to acute coronary syndrome. *Genetic testing and molecular biomarkers,* 16**,** 359-365.

STAMENKOVIC, I. 2003. Extracellular matrix remodelling: the role of matrix metalloproteinases. *The Journal of pathology,* 200**,** 448-464.

STOREY, E. 1973. The nature of tooth movement. *American journal of orthodontics,* 63**,** 292-314.

STRECKFUS, C. & BIGLER, L. 2002. Saliva as a diagnostic fluid. *Oral diseases,* 8**,** 69-76.

STURMER, T., RAUM, E., BUCHNER, M., GEBHARDT, K., SCHILTENWOLF, M., RICHTER, W. & BRENNER, H. 2005. Pain and high sensitivity C reactive protein in patients with chronic low back pain and acute sciatic pain. *Annals of the rheumatic diseases,* 64**,** 921-925.

SURESH, S., MAHENDRA, J. & SINGH, G. 2016. Comparative Analysis of GCF Resistin Levels in Obese Subjects with and without Periodontal Disease. *Group,* 2**,** 102.68.

SURLIN, P., RAUTEN, A. M., MOGOANTA, L., SILOSI, I., OPREA, B. & PIRICI, D. 2010. Correlations between the gingival crevicular fluid MMP8 levels and gingival overgrowth in patients with fixed orthodontic devices. *Romanian Journal of Morphology and Embryology,* 51**,** 515-9.

SUVAN, J., D'AIUTO, F., MOLES, D. R., PETRIE, A. & DONOS, N. 2011. Association between overweight/obesity and periodontitis in adults. A systematic review. *Obesity Reviews,* 12**,** e381-e404.

SUVAN, J., PETRIE, A., MOLES, D., NIBALI, L., PATEL, K., DARBAR, U., DONOS, N., TONETTI, M. & D'AIUTO, F. 2014. Body Mass Index as a predictive factor of periodontal therapy outcomes. *Journal of dental research,* 93**,** 49-54.

TABA, M., KINNEY, J., KIM, A. S. & GIANNOBILE, W. V. 2005. Diagnostic biomarkers for oral and periodontal diseases. *Dental Clinics of North America,* 49**,** 551-571.

TABARI, Z. A., AZADMEHR, A., NOHEKHAN, A., NADDAFPOUR, N. & GHAEDI, F. B. 2014. Salivary visfatin concentrations in patients with chronic periodontitis. *Journal of periodontology,* 85**,** 1081-1085.

TABARI, Z. A., AZADMEHR, A., TABRIZI, M. A. A., HAMISSI, J. & GHAEDI, F. B. 2013. Salivary soluble receptor activator of nuclear factor kappa B ligand/osteoprotegerin ratio in periodontal disease and health. *Journal of periodontal & implant science,* 43**,** 227-232.

TAKAHASHI, I., NISHIMURA, M., ONODERA, K., BAE, J.-W., MITANI, H., OKAZAKI, M. & SASANO, Y. 2003. Expression of MMP-8 and MMP-13 genes in the periodontal ligament during tooth movement in rats. *Journal of Dental Research,* 82**,** 646-651.

TAKAHASHI, T., ANZAI, T., KANEKO, H., MANO, Y., ANZAI, A., NAGAI, T., KOHNO, T., MAEKAWA, Y., YOSHIKAWA, T. & FUKUDA, K. 2010. Increased C-reactive protein expression exacerbates left ventricular dysfunction and remodeling after myocardial infarction. *American Journal of Physiology-Heart and Circulatory Physiology,* 299**,** H1795-H1804.

TALIC, N. F. 2011. Adverse effects of orthodontic treatment: a clinical perspective. *The Saudi dental journal,* 23**,** 55-59.

TAN, S., XIE, R., KLEIN-NULEND, J., VAN RHEDEN, R., BRONCKERS, A., KUIJPERS-JAGTMAN, A., VON DEN HOFF, J. & MALTHA, J. 2009. Orthodontic force stimulates eNOS and iNOS in rat osteocytes. *Journal of dental research,* 88**,** 255-260.

TANG, T. H., FITZSIMMONS, T. R. & BARTOLD, P. M. 2009. Effect of smoking on concentrations of receptor activator of nuclear factor κ B ligand and osteoprotegerin in human gingival crevicular fluid. *Journal of clinical periodontology,* 36**,** 713-718.

TANG, W. W., WU, Y., NICHOLLS, S. J. & HAZEN, S. L. 2011. Plasma myeloperoxidase predicts incident cardiovascular risks in stable patients undergoing medical management for coronary artery disease. *Clinical chemistry,* 57**,** 33-39.

TAUBMAN, M. A., VALVERDE, P., HAN, X. & KAWAI, T. 2005. Immune response: the key to bone resorption in periodontal disease. *Journal of periodontology,* 76**,** 2033-2041.

TAYEBJEE, M. H., LIP, G. Y., BLANN, A. D. & MACFADYEN, R. J. 2005. Effects of age, gender, ethnicity, diurnal variation and exercise on circulating levels of matrix metalloproteinases (MMP)-2 and-9, and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMP)-1 and-2. *Thrombosis research,* 115**,** 205-210.

TAYEBJEE, M. H., NADAR, S., BLANN, A. D., BEEVERS, D. G., MACFADYEN, R. J. & LIP, G. Y. 2004a. Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in hypertension and their relationship to cardiovascular risk and treatment: a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT). *American journal of hypertension,* 17**,** 764-769.

TAYEBJEE, M. H., NADAR, S. K., MACFADYEN, R. J. & LIP, G. Y. 2004b. Tissue inhibitor of metalloproteinase-1 and matrix metalloproteinase-9 levels in patients with hypertension: relationship to tissue Doppler indices of diastolic relaxation. *American journal of hypertension,* 17**,** 770-774.

TEITELBAUM, S. L. 2000. Bone resorption by osteoclasts. *Science,* 289**,** 1504-1508.

TELES, R., LIKHARI, V., SOCRANSKY, S. & HAFFAJEE, A. 2009. Salivary cytokine levels in subjects with chronic periodontitis and in periodontally healthy individuals: a cross-sectional study. *Journal of periodontal research,* 44**,** 411-417.

TERSIN, J. 1978. Studies of gingival conditions in relation to orthodontic treatment. IV. The effect of oral hygiene measures on gingival exudation during the course of orthodontic treatment. *Swedish dental journal,* 2**,** 131.

THE NHS INFORMATION CENTRE, L. S. 2012. Obesity among adults. *Statistics on obesity,physical activity and diet: Englnd, 2012.* UK.

TILG, H. & MOSCHEN, A. R. 2006. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nature Reviews Immunology,* 6**,** 772-783.

TIRANATHANAGUL, S., YONGCHAITRAKUL, T., PATTAMAPUN, K. & PAVASANT, P. 2004. Actinobacillus actinomycetemcomitans lipopolysaccharide activates matrix metalloproteinase-2 and increases receptor activator of nuclear factor-κB ligand expression in human periodontal ligament cells. *Journal of periodontology,* 75**,** 1647-1654.

TOBóN-ARROYAVE, S., JARAMILLO-GONZáLEZ, P. & ISAZA-GUZMáN, D. 2008. Correlation between salivary IL-1β levels and periodontal clinical status. *Archives of oral biology,* 53**,** 346-352.

TOBóN-ARROYAVE, S. I., ISAZA-GUZMáN, D. M., RESTREPO-CADAVID, E. M., ZAPATA-MOLINA, S. M. & MARTíNEZ-PABóN, M. C. 2012. Association of salivary levels of the bone remodelling regulators sRANKL and OPG with periodontal clinical status. *Journal of clinical periodontology,* 39**,** 1132-1140.

TODA, M. & MORIMOTO, K. 2008. Comparison of saliva sampling methods for measurement of salivary adiponectin levels. *Scandinavian journal of clinical and laboratory investigation,* 68**,** 823-825.

TODA, M., TSUKINOKI, R. & MORIMOTO, K. 2007. Measurement of salivary adiponectin levels. *Acta diabetologica,* 44**,** 20-22.

TOMS, S. R., LEMONS, J. E., BARTOLUCCI, A. A. & EBERHARDT, A. W. 2002. Nonlinear stress-strain behavior of periodontal ligament under orthodontic loading. *American journal of orthodontics and dentofacial orthopedics,* 122**,** 174-179.

TONG, H., RUDOLF, M., MUYOMBWE, T., DUGGAL, M. & BALMER, R. 2014. An investigation into the dental health of children with obesity: an analysis of dental erosion and caries status. *European Archives of Paediatric Dentistry,* 15**,** 203-210.

TORENSMA, B., THOMASSEN, I., VAN VELZEN, M. & ALEXANDER, B. 2016. Pain Experience and Perception in the Obese Subject Systematic Review (Revised Version). *Obesity surgery,* 26**,** 631-639.

TORNVIG, L., MOSEKILDE, L., JUSTESEN, J., FALK, E. & KASSEM, M. 2001. Troglitazone treatment increases bone marrow adipose tissue volume but does not affect trabecular bone volume in mice. *Calcified Tissue International,* 69**,** 46-50.

TOYGAR, H. U., KIRCELLI, B. H., BULUT, S., SEZGIN, N. & TASDELEN, B. 2008. Osteoprotegerin in gingival crevicular fluid under long-term continuous orthodontic force application. *The Angle Orthodontist,* 78**,** 988-993.

TROUVIN, A.-P. & GOEB, V. 2010. Receptor activator of nuclear factor-κB ligand and osteoprotegerin: maintaining the balance to prevent bone loss. *Clinical interventions in aging,* 5**,** 345.

TSICHLAKI, A., CHIN, S. Y., PANDIS, N. & FLEMING, P. S. 2016. How long does treatment with fixed orthodontic appliances last? A systematic review. *American Journal of Orthodontics and Dentofacial Orthopedics,* 149**,** 308-318.

TUNCER, B. B., ÖZDEMIR, B. Ç., BOYNUEĞRI, D., KARAKAYA, İ. B., ERGUDER, İ., YUCEL, A. A., ARAL, L. A. & ÖZMERIC, N. 2013. OPG-RANKL levels after continuous orthodontic force. *Gazi Medical Journal,* 24.

TUNCER, B. B., ÖZMERIC, N., TUNCER, C., TEOMAN, İ., ÇAKILCI, B., YUCEL, A., ALPAR, R. & BALOS, K. 2005. Levels of interleukin-8 during tooth movement. *The Angle orthodontist,* 75**,** 631-636.

TUNSTALL, A. M. & JAMES, K. 1974. Preliminary studies on the synthesis of alpha2-macroglobulin by human lymphocytes in vitro. *Clinical and experimental immunology,* 17**,** 697.

TURER, A. & SCHERER, P. 2012. Adiponectin: mechanistic insights and clinical implications. *Diabetologia,* 55**,** 2319-2326.

TURU, M. M., KRUPINSKI, J., CATENA, E., ROSELL, A., MONTANER, J., RUBIO, F., ALVAREZ-SABIN, J., CAIROLS, M. & BADIMON, L. 2006. Intraplaque MMP-8 levels are increased in asymptomatic patients with carotid plaque progression on ultrasound. *Atherosclerosis,* 187**,** 161-169.

TUTER, G., KURTIS, B. & SERDAR, M. 2007. Evaluation of gingival crevicular fluid and serum levels of high-sensitivity C-reactive protein in chronic periodontitis patients with or without coronary artery disease. *Journal of periodontology,* 78**,** 2319-2324.

TYROVOLA, J. B., PERREA, D., HALAZONETIS, D. J., DONTAS, I., VLACHOS, I. S. & MAKOU, M. 2010. Relation of soluble RANKL and osteoprotegerin levels in blood and gingival crevicular fluid to the degree of root resorption after orthodontic tooth movement. *Journal of oral science,* 52**,** 299-311.

TZANNETOU, S., EFSTRATIADIS, S., NICOLAY, O., GRBIC, J. & LAMSTER, I. 1999. Interleukin-1β and β-Glucuronidase in gingival crevicular fluid from molars during rapid palatal expansion. *American Journal of Orthodontics and Dentofacial Orthopedics,* 115**,** 686-696.

UDAGAWA, N., TAKAHASHI, N., JIMI, E., MATSUZAKI, K., TSURUKAI, T., ITOH, K., NAKAGAWA, N., YASUDA, H., GOTO, M. & TSUDA, E. 1999. Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor. *Bone,* 25**,** 517-523.

UEMATSU, S., MOGI, M. & DEGUCHI, T. 1996a. Increase of transforming growth factor-β 1 in gingival crevicular fluid during human orthodontic tooth movement. *Archives of oral biology,* 41**,** 1091-1095.

UEMATSU, S., MOGI, M. & DEGUCHI, T. 1996b. Interleukin (IL)-1β, IL-6, tumor necrosis factor-α, epidermal growth factor, and β2-microglobulin levels are elevated in gingival crevicular fluid during human orthodontic tooth movement. *Journal of Dental Research,* 75**,** 562-567.

UNQUEIRA, L. & CARNEIRO, J. 2005. Basic Histology: Text and Atlas. McGraw-Hill, New York.

URIBE, F., PADALA, S., ALLAREDDY, V. & NANDA, R. 2014. Patients', parents', and orthodontists' perceptions of the need for and costs of additional procedures to

reduce treatment time. *American Journal of Orthodontics and Dentofacial Orthopedics,* 145**,** S65-S73.

UZUNER, F. D. & DARENDELILER, N. 2013. Dentoalveolar surgery techniques combined with orthodontic treatment: A literature review. *European journal of dentistry,* 7**,** 257.

VALIATHAN, A. & DHAR, S. 2006. Prostaglandins and enhanced orthodontic tooth movement: In search of the silver bullet. *Current Science Bangalore* 90**,** 311.

VAN DIELEN, F., VAN'T VEER, C., SCHOLS, A., SOETERS, P., BUURMAN, W. & GREVE, J. 2001. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *International Journal of Obesity & Related Metabolic Disorders,* 25.

VAN NIEUW AMERONGEN, A., BOLSCHER, J. & VEERMAN, E. 2004. Salivary proteins: protective and diagnostic value in cariology? *Caries research,* 38**,** 247-253.

VARMA, M. C., KUSMINSKI, C. M., AZHARIAN, S., GILARDINI, L., KUMAR, S., INVITTI, C. & MCTERNAN, P. G. 2016. Metabolic endotoxaemia in childhood obesity. *BMC obesity,* 3**,** 1.

VERSTAPPEN, J. & VON DEN HOFF, J. W. 2006. Tissue inhibitors of metalloproteinases (TIMPs): their biological functions and involvement in oral disease. *Journal of dental research,* 85**,** 1074-1084.

VILJAKAINEN, H. T., PEKKINEN, M., SAARNIO, E., KARP, H., LAMBERG-ALLARDT, C. & MäKITIE, O. 2011. Dual effect of adipose tissue on bone health during growth. *Bone,* 48**,** 212-217.

VITOVSKI, S., PHILLIPS, J. S., SAYERS, J. & CROUCHER, P. I. 2007. Investigating the Interaction between Osteoprotegerin and Receptor Activator of NF-κB or Tumor Necrosis Factor-related Apoptosis-inducing Ligand EVIDENCE FOR A PIVOTAL ROLE FOR OSTEOPROTEGERIN IN REGULATING TWO DISTINCT PATHWAYS. *Journal of Biological Chemistry,* 282**,** 31601-31609.

VON BöHL, M., MALTHA, J. C., VON DEN HOFF, J. W. & KUIJPERS-JAGTMAN, A. M. 2004. Focal hyalinization during experimental tooth movement in beagle dogs. *American Journal of Orthodontics and Dentofacial Orthopedics,* 125**,** 615-623.

VON BREMEN, J., LORENZ, N. & RUF, S. 2015. Impact of body mass index on oral health during orthodontic treatment: an explorative pilot study. *The European Journal of Orthodontics***,** cjv074.

VON BREMEN, J., WAGNER, J. & RUF, S. 2012. Correlation between body mass index and orthodontic treatment outcome. *The Angle Orthodontist,* 83**,** 371-375.

VON ELM, E., ALTMAN, D. G., EGGER, M., POCOCK, S. J., GøTZSCHE, P. C. & VANDENBROUCKE, J. P. 2008. The Strengthening the Reporting of Observational Studies in Epidemiology [STROBE] statement: guidelines for reporting observational studies. *Gaceta Sanitaria,* 22**,** 144-150.

WAKI, H., YAMAUCHI, T., KAMON, J., ITO, Y., UCHIDA, S., KITA, S., HARA, K., HADA, Y., VASSEUR, F. & FROGUEL, P. 2003. Impaired multimerization of human adiponectin mutants associated with diabetes molecular structure and multimer formation of adiponectin. *Journal of Biological Chemistry,* 278**,** 40352-40363.

WALLACE, A. M., SATTAR, N. & MCMILLAN, D. C. 2000. The co-ordinated cytokine/hormone response to acute injury incorporates leptin. *Cytokine,* 12**,** 1042-1045.

WALTHER, D. P., HOUSTON, W. J. B., JONES, M. L. & OLIVER, R. G. 1994. *Walther and Houston's orthodontic notes*, John Wright.

WELLEN, K. E. & HOTAMISLIGIL, G. S. 2003. Obesity-induced inflammatory changes in adipose tissue. *Journal of Clinical Investigation,* 112**,** 1785.

WELTON, H. 2012. *The anatomy and physiology of salivary glands. In saliva and oral health. By Edgar W.M., Dawes C. and O'Mullane D.M,* UK, Stephen Hancocks Limited.

WHITE, R., JANOFF, A. & GODFREY, H. 1980. Secretion of alpha-2-macroglobulin by human alveolar macrophages. *Lung,* 158**,** 9-14.

WHITLOCK, G., LEWINGTON, S., SHERLIKER, P., CLARKE, R., EMBERSON, J., HALSEY, J., QIZILBASH, N., COLLINS, R. & PETO, R. 2009. Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. *Lancet,* 373**,** 1083-96.

WHO. 2013. *Growth Chart* [Online]. Available: www.rcpch.ac.uk/growthcharts [Accessed February 2014.

WILCZYŃSKA-BORAWSKA, M., BORAWSKI, J., BAGIŃSKA, J., MAŁYSZKO, J. & MYŚLIWIEC, M. 2012. Hepatocyte growth factor in saliva of patients with renal failure and periodontal disease. *Renal failure,* 34**,** 942-951.

WILCZYNSKA-BORAWSKA, M., BORAWSKI, J., KOVALCHUK, O., CHYCZEWSKI, L. & STOKOWSKA, W. 2006. Hepatocyte growth factor in saliva is a potential marker of symptomatic periodontal disease. *Journal of oral science,* 48**,** 47-50.

WILLENBROCK, F., CRABBE, T., SLOCOMBE, P. M., SUTTON, C. W., DOCHERTY, A. J., COCKETT, M. I., O'SHEA, M., BROCKLEHURST, K., PHILLIPS, I. R. & MURPHY, G. 1993. The activity of the tissue inhibitors of metalloproteinases is regulated by C-terminal domain interactions: a kinetic analysis of the inhibition of gelatinase A. *Biochemistry,* 32**,** 4330-4337.

WILLETT, W. C., MANSON, J. E., STAMPFER, M. J., COLDITZ, G. A., ROSNER, B., SPEIZER, F. E. & HENNEKENS, C. H. 1995. Weight, weight change, and coronary heart disease in women: risk within the'normal'weight range. *The Journal of the American Medical Association,* 273**,** 461-465.

WILLIAMSON, R. A., MARSTON, F., ANGAL, S., KOKLITIS, P., PANICO, M., MORRIS, H. R., CARNE, A., SMITH, B., HARRIS, T. & FREEDMAN, R. B. 1990. Disulphide bond assignment in human tissue inhibitor of metalloproteinases (TIMP). *Biochemical Journal,* 268**,** 267-274.

WILLIAMSON, S., MUNRO, C., PICKLER, R., GRAP, M. J. & ELSWICK, R. 2012. Comparison of biomarkers in blood and saliva in healthy adults. *Nursing research and practice,* 2012.

WISE, G. & KING, G. 2008. Mechanisms of tooth eruption and orthodontic tooth movement. *Journal of dental research,* 87**,** 414-434.

WŁODARSKI, K. & WŁODARSKI, P. 2008. Leptin as a modulator of osteogenesis. *Ortopedia, traumatologia, rehabilitacja,* 11**,** 1-6.

WONG, D. T. 2012. Salivaomics. *The Journal of the American Dental Association,* 143**,** 19S-24S.

WONG, G. W., WANG, J., HUG, C., TSAO, T.-S. & LODISH, H. F. 2004. A family of Acrp30/adiponectin structural and functional paralogs. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 10302-10307.

WONG, H., COLLINS, J., TINSLEY, D., SANDLER, J. & BENSON, P. 2013. Does the bracket–ligature combination affect the amount of orthodontic space closure over three months? A randomized controlled trial. *Journal of orthodontics,* 40**,** 155-162.

WOODHOUSE, N., DIBIASE, A., JOHNSON, N., SLIPPER, C., GRANT, J., ALSALEH, M., DONALDSON, A. & COBOURNE, M. 2015a. Supplemental Vibrational Force During Orthodontic Alignment A Randomized Trial. *Journal of dental research,* 94**,** 682-689.

WOODHOUSE, N. R., DIBIASE, A. T., PAPAGEORGIOU, S. N., JOHNSON, N., SLIPPER, C., GRANT, J., ALSALEH, M. & COBOURNE, M. T. 2015b. Supplemental vibrational force does not reduce pain experience during initial alignment with fixed orthodontic appliances: a multicenter randomized clinical trial. *Scientific reports,* 5.

XIE, R., KUIJPERS-JAGTMAN, A. M. & MALTHA, J. C. 2008. Osteoclast differentiation during experimental tooth movement by a short-term force application: an immunohistochemical study in rats. *Acta Odontologica Scandinavica,* 66**,** 314-320.

XU, F., DU, Y., HANG, S., CHEN, A., GUO, F. & XU, T. 2013. Adipocytes regulate the bone marrow microenvironment in a mouse model of obesity. *Molecular medicine reports,* 8**,** 823-828.

YAMAGUCHI, M. 2009. RANK/RANKL/OPG during orthodontic tooth movement. *Orthodontics & craniofacial research,* 12**,** 113-119.

YAMAGUCHI, M. & KASAI, K. 2005. Inflammation in periodontal tissues in response to mechanical forces. *Archivum Immunologiae et Therapiae Experimentalis -English Edittion* 53**,** 388.

YAMAGUCHI, M., NISHIMURA, F., NARUISHI, H., SOGA, Y., KOKEGUCHI, S. & TAKASHIBA, S. 2005. Thiazolidinedione (pioglitazone) blocks P. gingivalis-and F. nucleatum, but not E. coli, lipopolysaccharide (LPS)-induced interleukin-6 (IL-6) production in adipocytes. *Journal of dental research,* 84**,** 240-244.

YAMAGUCHI, N., HAMACHI, T., KAMIO, N., AKIFUSA, S., MASUDA, K., NAKAMURA, Y., NONAKA, K., MAEDA, K., HANAZAWA, S. & YAMASHITA, Y. 2010. Expression levels of adiponectin receptors and periodontitis. *Journal of periodontal research,* 45**,** 296-300.

YAMALIK, N., ÇAGLAYAN, F., KILINC, K., KILINC, A. & TUMER, C. 2000. The importance of data presentation regarding gingival crevicular fluid myeloperoxidase and elastase-like activity in periodontal disease and health status. *Journal of Periodontology,* 71**,** 460-467.

YAMASAKI, K., SHIBATA, Y., IMAI, S., TANI, Y., SHIBASAKI, Y. & FUKUHARA, T. 1984. Clinical application of prostaglandin E 1 (PGE 1) upon orthodontic tooth movement. *American journal of orthodontics,* 85**,** 508-518.

YAMASHITA, J. M., DE MOURA-GREC, P. G., DE FREITAS, A. R., SALES-PERES, A., GROPPO, F. C., CENEVIVA, R. & DE CARVALHO SALES-PERES, S. H. 2015. Assessment of Oral Conditions and Quality of Life in Morbid Obese and Normal Weight Individuals: A Cross-Sectional Study. *PloS one,* 10**,** e0129687.

YAMAUCHI, T., KAMON, J., ITO, Y., TSUCHIDA, A., YOKOMIZO, T., KITA, S., SUGIYAMA, T., MIYAGISHI, M., HARA, K. & TSUNODA, M. 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature,* 423**,** 762-769.

YAMAUCHI, T., KAMON, J., MINOKOSHI, Y. A., ITO, Y., WAKI, H., UCHIDA, S., YAMASHITA, S., NODA, M., KITA, S. & UEKI, K. 2002. Adiponectin stimulates

glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature medicine,* 8**,** 1288-1295.

YANG, T.-T. & HAWKES, S. P. 1992. Role of the 21-kDa protein TIMP-3 in oncogenic transformation of cultured chicken embryo fibroblasts. *Proceedings of the National Academy of Sciences,* 89**,** 10676-10680.

YAO, Y., FENG, X. & JING, X. 2003. [The correlation between tooth pain and bioactivators changes in gingival crevicular fluid after applying orthodontic stress]. *Shanghai kou qiang yi xue= Shanghai journal of stomatology,* 12**,** 331-333.

YASUDA, H., SHIMA, N., NAKAGAWA, N., YAMAGUCHI, K., KINOSAKI, M., GOTO, M., MOCHIZUKI, S.-I., TSUDA, E., MORINAGA, T. & UDAGAWA, N. 1999. A novel molecular mechanism modulating osteoclast differentiation and function. *Bone,* 25**,** 109-113.

YAZID, M. D., ARIFFIN, S., SENAFI, S., RAZAK, M. A. & WAHAB, R. 2010. Determination of the differentiation capacities of murines' primary mononucleated cells and MC3T3-E1 cells. *Cancer Cell Int,* 10**,** 42.

YI, J., YAN, B., LI, M., WANG, Y., ZHENG, W., LI, Y. & ZHAO, Z. 2016. Caffeine may enhance orthodontic tooth movement through increasing osteoclastogenesis induced by periodontal ligament cells under compression. *Archives of oral biology,* 64**,** 51-60.

YIN, J., GAO, H., YANG, J., XU, L. & LI, M. 2012. Measurement of salivary resistin level in patients with type 2 diabetes. *International journal of endocrinology,* 2012.

YLöSTALO, P., SUOMINEN-TAIPALE, L., REUNANEN, A. & KNUUTTILA, M. 2008. Association between body weight and periodontal infection. *Journal of Clinical Periodontology,* 35**,** 297-304.

YOON, M. J., LEE, G. Y., CHUNG, J.-J., AHN, Y. H., HONG, S. H. & KIM, J. B. 2006. Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator–activated receptor α. *Diabetes,* 55**,** 2562-2570.

ZAHORSKA-MARKIEWICZ, B., KUCIO, C. & PYSZKOWSKA, J. 1983. Obesity and pain. *Human nutrition. Clinical nutrition,* 37**,** 307.

ZAHORSKA-MARKIEWICZ, B., ZYCH, P. & KUCIO, C. 1988. Pain sensitivity in obesity. *Acta Physiologica Polonica,* 39**,** 183.

ZAINAL ARIFFIN, S. H., YAMAMOTO, Z., MEGAT ABDUL WAHAB, R. & ZAINAL ARIFFIN, Z. 2011. Cellular and molecular changes in orthodontic tooth movement. *The Scientific World Journal,* 11**,** 1788-1803.

ZEIGLER, C. C., PERSSON, G. R., WONDIMU, B., MARCUS, C., SOBKO, T. & MODEER, T. 2012. Microbiota in the oral subgingival biofilm is associated with obesity in adolescence. *Obesity,* 20**,** 157-164.

ZETU, I., OGODESCU, E., ZETU, L., STRATUL, S., RUSU, D., TALPOŞ, S. & OGODESCU, A. 2010. [An interdisciplinary approach to complex orthodontic-periodontal clinical situations in adult patients]. *Revista medico-chirurgicala a Societatii de Medici si Naturalisti din Iasi,* 115**,** 1262-1266.

ZHANG, S., LIU, Q., WANG, J. & HARNISH, D. C. 2009. Suppression of interleukin-6-induced C-reactive protein expression by FXR agonists. *Biochemical and biophysical research communications,* 379**,** 476-479.

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 372**,** 425-432.

ZHOU, B., LIU, J., WANG, Z.-M. & XI, T. 2012. C-reactive protein, interleukin 6 and lung cancer risk: a meta-analysis. *PloS one,* 7**,** e43075.

ZHOU, Y. & RUI, L. 2013. Leptin signaling and leptin resistance. *Frontiers of medicine,* 7**,** 207-222.

ZIA, A., KHAN, S., BEY, A., GUPTA, N. & MUKHTAR-UN-NISAR, S. 2011. Oral biomarkers in the diagnosis and progression of periodontal diseases. *Biology and Medicine,* 3**,** 45-52.

ZIMMERMANN, G. S., BASTOS, M. F., DIAS GONCALVES, T. E., CHAMBRONE, L. & DUARTE, P. M. 2013. Local and circulating levels of adipocytokines in obese and normal weight individuals with chronic periodontitis. *Journal of periodontology,* 84**,** 624-633.

Journal of Dental Research
1–8

DOI: 10.1177/0022034516688448
journals.sagepub.com/home/jdr

# Impact of Obesity on Orthodontic Tooth Movement in Adolescents: A Prospective Clinical Cohort Study

**H.F. Saloom[1,2,3], S.N. Papageorgiou[4], G.H. Carpenter[2], and M.T. Cobourne[1]**

**Abstract**

Obesity is a widespread chronic inflammatory disorder characterized by an increased overall disease burden and significant association with periodontitis. The aim of this prospective clinical cohort study was to investigate the effect of obesity on orthodontic tooth movement. Fifty-five adolescent patients (27 males, 28 females) with a mean (SD) age of 15.1 (1.7) years and mean (SD) body mass index (BMI) of 30.2 (3.5) kg/m$^2$ in obese and 19.4 (2.2) kg/m$^2$ in normal-weight groups were followed from start of treatment to completion of tooth alignment with fixed orthodontic appliances. Primary outcome was time taken to complete tooth alignment, while secondary outcomes included rate of tooth movement and change in clinical parameters (plaque/gingival indices, unstimulated whole-mouth salivary flow rate, gingival crevicular fluid biomarkers). Data collection took place at baseline (start of treatment: appliance placement), 1 h and 1 wk following appliance placement, and completion of alignment. Results were analyzed by descriptive statistics followed by generalized estimating equation regression modeling. There were no significant differences between groups in time taken to achieve tooth alignment (mean [SD] 158.7 [75.3] d; $P = 0.486$). However, at 1 wk, initial tooth displacement was significantly increased in the obese group ($P < 0.001$), and after adjusting for confounders, obese patients had a significantly higher rate of tooth movement compared with normal-weight patients (+0.017 mm/d; 95% confidence interval, 0.008–0.025; $P < 0.001$) over the period of alignment. Explorative analyses indicated that levels of the adipokines leptin and resistin, the inflammatory marker myeloperoxidase (MPO), and the cytokine receptor for nuclear factor kappa-B ligand (RANKL) were significantly different between obese- and normal-weight patients and associated with observed rates of tooth movement. This represents the first prospective data demonstrating a different response in obese patients compared with normal-weight patients during early orthodontic treatment. These differences in the response of periodontal tissues to orthodontic force in the presence of obesity have potential short- and long-term clinical implications.

**Keywords:** biomarkers, BMI, inflammation, MMPs, RANKL, saliva

## Introduction

Obesity levels have been rising among children and adults in Western societies over the past few decades (Ng et al. 2014). This represents a major health care challenge because of the known associations between raised body mass index (BMI) and multiple chronic diseases, including insulin-resistant diabetes, cardiovascular disease, and cancer (Deng et al. 2016). It is recognized that obesity represents a state of chronic subclinical inflammation mediated through excess adipose tissue (Hotamisligil 2006). Adipocytes produce a host of metabolically active proteins or adipokines that influence metabolic function and inflammatory responses (Ouchi et al. 2011) and include proinflammatory leptin (Zhang et al. 1994) and resistin (Steppan et al. 2001) and anti-inflammatory adiponectin (Scherer et al. 1995).

Adipose tissue can influence the intensity and resolution of inflammatory responses in multiple tissues (Issa and Griffin 2012; Pierpont et al. 2014). Indeed, an increased risk of chronic periodontitis (Suvan et al. 2011; Keller et al. 2015) and variation in inflammatory and metabolic markers exist in obese subjects affected by periodontal disease compared with normal-weight subjects (Papageorgiou, Reichert, et al. 2015). The systemic induction of inflammatory markers may provide a link between obesity and periodontitis, with current focus on C-reactive

[1]Department of Orthodontics, King's College London Dental Institute, London, UK
[2]Department of Mucosal and Salivary Biology, King's College London Dental Institute, London, UK
[3]Department of Orthodontics, College of Dentistry, University of Baghdad, Baghdad, Iraq
[4]Clinic of Orthodontics and Pediatric Dentistry, Center of Dental Medicine, University of Zurich, Zurich, Switzerland

A supplemental appendix to this article is available online.

**Corresponding Author:**
M.T. Cobourne, Department of Craniofacial Development and Orthodontics, King's College London Dental Institute, Floor 27, Guy's Hospital, London SE1 9RT, UK.
Email: martyn.cobourne@kcl.ac.uk

protein (CRP) as an important potential mediator (Pradeep et al. 2012).

Orthodontic tooth movement is initially represented by simple mechanical displacement of the tooth and bone bending within the socket, which occurs due to compression of the periodontal ligament following the application of external force. However, over the longer term, tooth movement occurs as a direct consequence of connective tissue remodeling within the periodontium and alveolar bone mediated through a localized inflammatory response. This triggers the release of essential biochemical mediators, which are often detectable within gingival crevicular fluid (GCF) (Kapoor et al. 2014)—in particular, the tissue-modulating factors receptor for nuclear factor kappa-B ligand (RANKL), matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs) (Grant et al. 2013). Obesity is also known to influence systemic bone metabolism through complex mechanical, hormonal, and inflammatory interactions (López-Gómez et al. 2016) with associations between obesity and reduced bone remodeling (Ivaska et al. 2016) and increased bone mineral density (Salamat et al. 2016). Although there are little data relating obesity to changes in alveolar bone composition within the healthy periodontium, longitudinal data have shown a significant association with increased rates of tooth eruption (Must et al. 2012).

The potential implications of adolescent obesity for orthodontic treatment have been highlighted (Neeley and Gonzales 2007), with increased BMI a risk factor for less cooperation and longer treatment duration with fixed appliances (von Bremen et al. 2016). However, despite known associations between raised BMI and chronic inflammatory changes within the periodontium, there have been no prospective investigations of orthodontic tooth movement in obese patients. The aim of this study was to investigate the effect of obesity on orthodontic tooth movement during routine treatment with fixed appliances—specifically, time taken to complete tooth alignment and variation in clinical parameters, including GCF biomarkers.

## Materials and Methods

### Study Design

This prospective cohort study compared the effects of obesity on tooth alignment with fixed appliances. Ethical approval was obtained from the United Kingdom National Research Ethics Service (UK NRES) (14/LO/0769). Written informed consent was received from all parents, guardians, and children. We report and present data according to STROBE (Strengthening the Reporting of Observational Studies in Epidemiology; von Elm et al. 2008).

### Setting

Participants fulfilling the inclusion criteria were recruited consecutively from orthodontic treatment clinics at King's College London Dental Institute (Guy's Hospital) between January 2015 and January 2016. Follow-up occurred to June 2016 and covered appliance placement to completion of alignment.

### Participants

Inclusion criteria included the following: 1) fixed-appliance treatment, 2) aged 12 to 18 y at treatment start, 3) no medical contraindications or regular medication (including antibiotic therapy) in the last 6 mo, 4) nonsmokers, 5) permanent dentition, 6) mandibular arch incisor irregularity index of 4 to 12 mm, and 7) normal-weight (BMI-centile 2–91) and obese (BMI-centile >98) classification. Those classified as underweight (BMI-centile <2) and overweight (BMI-centile 91–98) were excluded, respectively.

Subject body weight was measured to the nearest 0.1 kg using a calibrated scale and height measured to the nearest centimeter using a wall-mounted rule. BMI was calculated as mass (kg) divided by height in meters squared ($kg/m^2$). United Kingdom Royal College of Pediatrics and Child Health World Health Organization growth charts were used to calculate and classify BMI-centile in relation to age and sex (World Health Organization/Royal College of Paediatrics and Child Health 2016). All measurements were taken by a single-trained operator (H.F.S.) using the same equipment.

### Variables

Tooth alignment was calculated from scanned (3Shape-R700) stone dental casts using an irregularity index (Little 1975).

Unstimulated whole mouth salivary flow rate (uWMS) was calculated as milliliter per minute from saliva obtained from relaxed patients spitting into a plastic tube for 5 min. Periodontal health was measured clinically using established validated plaque and gingival indices (Loe and Silness 1963; Silness and Loe 1964). GCF was collected from the distal side of the lower 6 anterior teeth and pooled. Following isolation, teeth were gently dried using an air syringe and Periopaper filter strips (OraFlow) placed 1 mm into the gingival crevice for 30 s. If there was any contamination of the strip with saliva or blood, it was discarded. The volume of collected fluid was measured directly using a Periotron-8000 electronic micromoisture meter (OraFlow) with readings converted to an actual volume by reference to the standard curve and flow rate calculated (per minute). GCF was retrieved from filter strips with the addition of 20 µL phosphate-buffered saline (PBS) and centrifugation for 5 min at 9,200 × *g*. Samples were stored at −80°C for subsequent analysis. GCF was analyzed by a Luminex bead-based multiplex assay using a commercially available kit (R&D Systems) for detection (pg/mL) of adiponectin, leptin, and resistin; inflammatory mediators myeloperoxidase (MPO) and CRP; and tissue-remodeling biomarkers MMP8, MMP9, TIMP1, MMP8/TIMP1, MMP9/TIMP1, and RANKL (Appendix Table 1).

Fixed appliances and bonding method were standardized (3M Victory-APC 0.022-inch brackets, MBT prescription; 3M-Unitek). After bracket bonding, a 0.014-inch nickel-titanium archwire was tied in and ligated using conventional elastomerics. The archwire was cut distal to the first molar teeth and not cinched. No bite planes, auxiliary arches, intermaxillary elastics, headgears, or temporary anchorage devices

were used during the investigation. All appliances were placed by postgraduate orthodontic trainees under direct supervision of a consultant orthodontist.

Sample collection took place during routine appointments between 9:30 a.m. and 3:30 p.m. at baseline (start of treatment: appliance placement) (irregularity index, uWMS, plaque and gingival indices, GCF flow rate, biomarker analysis), 1 h following appliance placement (GCF flow rate, biomarker analysis), 1 wk following appliance placement (irregularity index, uWMS, plaque and gingival indices, GCF flow rate, biomarker analysis), and completion of tooth alignment (0.019 × 0.025-inch stainless steel archwire) (irregularity index, uWMS, plaque and gingival indices, GCF flow rate, biomarker analysis). This was a pragmatic study undertaken in a clinical department. Patients were seen at approximate 6-wk intervals for appliance adjustment, and archwire progression took place as deemed clinically appropriate by treating clinicians. Patient flow through the study is shown in the Appendix Figure.

Primary outcome was time to achieve tooth alignment in the lower arch. Secondary outcomes included rate of tooth movement, changes in clinical parameters, and GCF biomarkers during treatment. There were no changes to outcomes following study commencement.

### *Sample Size*

Sample size was based on previous randomized prospective data on time to completion of alignment with fixed appliances, which found a mean (SD) time to alignment of 200.7 (73.6) d in the presence of an 8.9-mm incisor irregularity (Woodhouse et al. 2015). A total of 50 patients were required to detect with an unpaired *t* test a hypothesized 30% reduction (Schulz and Grimes 2005) in alignment time with a common SD across groups to yield 80% power ($P = 0.05$). Five additional patients were recruited to account for possible dropouts.

### *Statistical Methods*

Descriptive statistics were calculated after checking for normal distribution. All biomarker data were $\log_{10}$ transformed for normalization. Initial crude differences in baseline and outcome data were calculated with independent *t* tests, $\chi^2$ tests, or Mann-Whitney tests, where needed.

The effect of obesity was investigated using univariable (crude) and multivariable generalized estimation equation regression models with robust standard errors to take into account correlation between repeated measurements for each patient through the follow-up period (baseline, 1 h, 1 wk, and completion of alignment), adjusted for the confounding effect of baseline data (sex, age, baseline irregularity). Results are reported as unstandardized coefficients or odds ratios for continuous and binary outcomes, respectively. The effect modification of obesity on the progress of tooth alignment was tested by introducing interaction terms, which were ultimately dropped from the model, if not significant. Analysis of residuals was conducted to confirm the regression assumptions. As patients within the study had initial irregularity ranging from 4 to 12 mm, sensitivity analyses were conducted to include only those with severe (≥7 mm) or moderate (4–7 mm) baseline irregularity. All analyses were carried out using Stata 12.0 (StataCorp LP). A 2-tailed *P* value of 0.05 was considered statistically significant with a 95% confidence interval (CI) for all tests.

All primary data were coded so that the outcome assessor (H.F.S.) and statistician (S.N.P.) were blinded to subject classification. Data coding was broken following analysis, and no blinding breaches were identified. To examine measurement reliability and agreement, 36 pairs of models from baseline and 1 wk were selected and remeasured after 2 wk. The concordance correlation coefficient (CCC) (Lin 1989) and Bland-Altman method (Bland and Altman 1986) were used to test intraexaminer reliability and agreement.

## Results

### *Participants*

This study included 55 patients (27 male, 28 female) with a mean (SD) age of 15.1 (1.7) years and a mean (SD) irregularity index of 7.6 (2.4) mm. Mean (SD) BMI of the cohort was 24.7 (6.2) kg/m$^2$. From the original 55 patients recruited, 7 were excluded at 1 wk due to missed appointments, but all 55 were included at completion of alignment. Missingness at 1 wk was judged as random (Appendix Table 2). The reliability and agreement of repeated measurements were excellent (CCC = 0.99 with 95% CI, 0.98–0.99; average difference of 0.06 with 95% limits of agreement, –0.68 to 0.79). Table 1 shows demographics and GCF parameters for the 2 cohorts at baseline. Mean (SD) BMI was 19.4 (2.2) in the normal-weight group and 30.2 (3.5) kg/m$^2$ in the obese group. Apart from BMI, there were no statistically significant differences in demographics among groups at baseline; however, the obese group did have 1.2 mm more irregularity ($P = 0.061$). In contrast, significant differences were present between normal-weight and obese groups for a number of GCF biomarkers at baseline (Table 1; $P < 0.05$), including increased GCF flow rate; increased leptin, resistin, MPO, MMP8, TIMP1, and RANKL; and reduced MMP9/TIMP1 levels in obese patients.

### *Primary Outcome*

The results of both crude and adjusted regression analyses indicated a small difference in time required to achieve tooth alignment between obese and normal-weight patients. Overall, obese patients needed a mean 23.0 d less than normal-weight patients to reach final alignment (Fig. A), but this was not statistically significant (Table 2, $P > 0.05$).

### *Secondary Outcomes*

A number of significant differences were observed between obese and normal-weight patients in the clinical response to

**Table 1.** Demographics and GCF Parameters of Patients at Baseline.

| | Overall | Normal Weight | Obese | *P* Value |
|---|---|---|---|---|
| Demographics | | | | |
| Patients (*n*) | 55 | 28 | 27 | |
| Male/female (*n*) | 27/28 | 15/13 | 12/15 | 0.498[a] |
| Age, y | 15.1 (1.7) | 15.1 (1.6) | 15.1 (1.9) | 0.991[b] |
| Race, *n* (%) | | | | |
| Caucasian | 29 (53) | 17 (61) | 12 (44) | 0.516[a] |
| Asian | 4 (7) | 2 (7) | 2 (7) | |
| African | 5 (9) | 1 (4) | 4 (15) | |
| Mixed | 8 (15) | 3 (11) | 5 (19) | |
| Other | 9 (16) | 5 (18) | 4 (15) | |
| BMI, kg/m$^2$ | 24.7 (6.2) | 19.4 (2.2) | 30.2 (3.5) | **<0.001**[b] |
| uWMS, mL/min | 0.61 (0.32) | 0.64 (0.34) | 0.58 (0.29) | 0.480[b] |
| Plaque index | 0.56 (0.32) | 0.57 (0.32) | 0.54 (0.31) | 0.745[b] |
| Gingival index | 0.74 (0.39) | 0.74 (0.40) | 0.73 (0.38) | 0.934[b] |
| Irregularity index, mm | 7.6 (2.4) | 7.0 (2.3) | 8.2 (2.4) | 0.061[b] |
| Severe irregularity, *n* (%) | 31 (56) | 13 (46) | 18 (67) | 0.130[a] |
| Tooth extraction, *n* (%) | 8 (15) | 4 (14) | 4 (15) | 0.956[a] |
| GCF biomarkers, pg/mL($\log_{10}$ transformed[c]) | | | | |
| GCF flow rate, µL/min | –0.29 (0.14) | –0.33 (0.12) | –0.25 (0.12) | **0.011**[d] |
| Adiponectin | 6.60 (0.34) | 6.55 (0.42) | 6.66 (0.23) | 0.237[b] |
| Leptin[e] | 13.91 (22.64) | 6.40 (14.65) | 19.15 (24.45) | **0.031**[d] |
| Resistin | 5.61 (0.56) | 5.30 (0.55) | 5.92 (0.36) | **<0.001**[b] |
| MPO | 5.05 (0.85) | 4.44 (0.64) | 5.69 (0.52) | **<0.001**[b] |
| CRP | 2.50 (0.51) | 2.47 (0.27) | 2.65 (0.87) | 0.827[b] |
| MMP8 | 6.32 (0.64) | 6.01 (0.48) | 6.64 (0.64) | **<0.001**[b] |
| MMP9 | 6.23 (0.30) | 6.18 (0.59) | 6.27 (0.15) | 0.245[b] |
| TIMP1 | 5.00 (0.48) | 4.72 (0.38) | 5.28 (0.40) | **<0.001**[b] |
| MMP8/TIMP1 | 0.96 (0.64) | 0.93 (0.62) | 0.99 (0.68) | 0.699[b] |
| MMP9/TIMP1 | 0.54 (0.54) | 0.75 (0.54) | 0.32 (0.47) | **0.002**[b] |
| RANKL | 3.54 (0.33) | 3.39 (0.25) | 3.65 (0.27) | **<0.001**[d] |

For demographics: values are mean (SD) unless otherwise indicated. For GCF parameters: values are pg/mL unless otherwise indicated. Significant results are indicated in bold. BMI, body mass index; CRP, C-reactive protein; GCF, gingival crevicular fluid; MMP8, matrix metalloproteinase 8; MMP9, matrix metalloproteinase 9; MPO, myeloperoxidase; RANKL, receptor activator of nuclear factor kappa-B ligand; TIMP1, tissue inhibitor of metalloproteinase 1; uWMS, unstimulated whole-mouth salivary flow rate.

[a]From $\chi^2$ test.

[b]From independent *t* test.

[c]$\log_{10}$ transformation improved the skewness of the data, but the Shapiro-Wilk test indicated that transformed data were still not normally distributed. Therefore, the median (interquartile range) is presented instead of mean (SD) and the Mann-Whitney test is used on the transformed data instead of the unpaired *t* test.

[d]From Mann-Whitney test.

[e]Square root transformation was used instead of $\log_{10}$, as several null values were included. Therefore, the median (interquartile range) is presented instead of mean (SD) and the Mann-Whitney test is used on the transformed data instead of the unpaired *t* test.

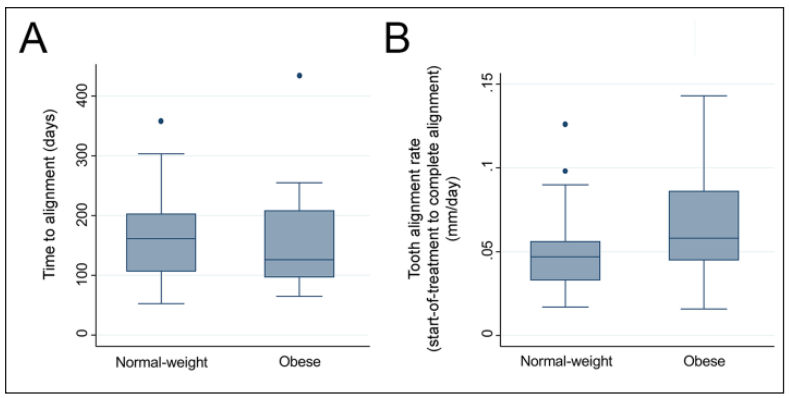



**Figure.** Box plots of measured values in normal-weight and obese patients for (**A**) primary (time to completion of alignment in days; left panel) and (**B**) secondary (tooth alignment rate from start of treatment to completion of alignment in mm/d; right panel) outcomes. Plotted boxes with horizontal lines indicate interquartile ranges with medians. Vertical whiskers and points indicate upper and lower adjacent values and outliers.

orthodontic force. The rate of mechanical tooth displacement within week 1 was significantly increased in the obese group ($P < 0.001$), while overall rate of alignment from baseline to completion of alignment was also increased ($P = 0.05$) (Table 3). However, tooth alignment rate from week 1 to completion of alignment was not significantly different between groups (Table 3; $P = 0.119$). After taking into account all confounders in the adjusted analysis, obese patients were associated with a significantly increased rate of tooth movement throughout the whole study duration compared with normal-weight patients (0.017 mm/d; 95% CI, 0.008–0.025 mm/d; $P < 0.001$) (Fig. B). In addition, a significant association was found between rate of tooth movement and initial irregularity (0.007mm/d increase per millimeter of irregularity). Sensitivity

**Table 2.** Regression Analysis on Primary Outcome (Time to Completion of Alignment in Days) and Secondary Outcome (Rate of Orthodontic Tooth Movement in mm/d).

| | Crude Model | | | Adjusted Model | | |
|---|---|---|---|---|---|---|
| Factor | b | 95% CI | *P* Value | b | 95% CI | *P* Value |
| Primary outcome (time to completion of tooth alignment) | | | | | | |
| BMI group | | | | | | |
| Obese | −14.3 | −54.3 to 25.7 | 0.483 | −23.0 | −66.1 to 20.1 | 0.295 |
| Control | Reference | | | | | |
| Age | | | | | | |
| Per year | NT | | | −3.2 | −15.6 to 9.1 | 0.608 |
| Sex | | | | | | |
| Male | NT | | | −6.6 | −48.4 to 35.2 | 0.756 |
| Female | NT | | | | | |
| Ethnicity | NT | | | −0.2 | −13.4 to 13.0 | 0.974 |
| Extraction | | | | | | |
| Yes | NT | | | −15.3 | −76.0 to 45.5 | 0.623 |
| No | NT | | | | | |
| Baseline irregularity, per mm | NT | | | 6.9 | −2.5 to 16.3 | 0.151 |
| Secondary outcome (rate of orthodontic tooth movement: baseline to completion of alignment) | | | | | | |
| BMI group | | | | | | |
| Obese | 0.023 | 0.011 to 0.035 | <0.001 | 0.017 | 0.008 to 0.025 | **<0.001** |
| Control | Reference | | | Reference | | |
| Time | | | | | | |
| 1 wk | Reference | | | Reference | | |
| Complete alignment | −0.025 | −0.036 to 0.015 | <0.001 | −0.021 | −0.032 to −0.010 | **<0.001** |
| Age, per year | NT | | | 0.001 | −0.002 to 0.003 | 0.609 |
| Sex | | | | | | |
| Male | NT | | | 0.001 | −0.008 to 0.011 | 0.768 |
| Female | NT | | | Reference | | |
| Ethnicity | NT | | | −0.001 | −0.003 to 0.002 | 0.643 |
| Irregularity at each phase start, per mm | NT | | | 0.007 | 0.005 to 0.009 | **<0.001** |
| Extraction | | | | | | |
| Yes | NT | | | −0.004 | −0.014 to 0.006 | 0.415 |
| No | NT | | | Reference | | |

Interaction of obesity with time found to be nonsignificant ($P = 0.112$) and was dropped from the model. Significant results are indicated in bold. b, unstandardized regression coefficient; CI, confidence interval; BMI, body mass index; NT, not tested.

analyses for patients with either severe or moderate irregularity were consistent in direction with the main analysis (Appendix Tables 3 and 4, respectively), with an expected loss of power due to the division of the study sample and a higher difference in alignment rate between obese and normal-weight patients in the severe irregularity group.

uWMS increased during treatment, while plaque and gingival indices deteriorated significantly (Appendix Table 5), but there were no differences between groups for either of these parameters. GCF flow rate increased during orthodontic treatment for both groups, but significantly more in obese patients.

To further understand the biochemical basis of observed differences in rates of tooth movement, explorative regression analyses were undertaken (Table 4). GCF levels of leptin, resistin, MPO, MMP8, TIMP1, MMP9/TIMP1, and RANKL were significantly different between obese and normal-weight patients at baseline and during subsequent assays (Appendix Table 5). When a possible interrelation between these biomarkers and rate of tooth movement was investigated, it was found that leptin, resistin, MPO, and RANKL were significantly associated with the amount of tooth movement for each patient (Appendix Table 6). Therefore, from an epidemiological basis, these biomarkers are the best candidates to explain the clinical performance difference between obese and normal-weight patients during orthodontic tooth alignment with fixed appliances.

## Discussion

This prospective study followed a cohort of obese and normal-weight adolescent patients during the alignment phase of fixed-appliance orthodontic treatment. Obese patients demonstrated significantly increased rates of tooth movement during the whole observation period, although there were no significant differences in time taken to achieve alignment. This apparent discrepancy might be explained by a number of factors. First, the obese group had a significantly increased initial mechanical displacement of the teeth during the first week following the application of orthodontic force, there was also a slightly increased (albeit statistically nonsignificant) baseline irregularity present in the obese group, and there may have been possible between-group variation in attendance during routine appointments. Evidence exists from a similar experimental

**Table 3.** Rate of Tooth Movement (mm/d) during the Study Period.

| Outcome | Overall ($n$ = 55), Mean (SD) | Normal Weight ($n$ = 28), Mean (SD) | Obese ($n$ = 27), Mean (SD) | $P$ Value |
|---|---|---|---|---|
| Time to completion of alignment (d) | 158.7 (75.3) | 165.8 (72.5) | 151.4 (78.7) | 0.486 |
| Tooth alignment rate: baseline to completion of alignment (mm/d) | 0.057 (0.029) | 0.050 (0.025) | 0.065 (0.031) | **0.050**[a] |
| Initial tooth displacement rate: baseline to week 1 (mm/d)[b] | 0.081 (0.031) | 0.065 (0.025) | 0.097 (0.028) | **<0.001**[a] |
| Tooth alignment rate: week 1 to completion of alignment (mm/d)[b] | 0.056 (0.031) | 0.049 (0.027) | 0.063 (0.033) | 0.119[a] |

CI, confidence interval; SD, standard deviation.
[a]From independent *t* test.
[b]Due to 7 patient dropouts at 1 wk, 48 of 55 patients (24 obese and 24 control patients) are included in these 2 measurements. The measurement of time to completion of alignment and alignment rate: baseline to completion of alignment pertain to the whole sample of 55 patients.

**Table 4.** Summary of Exploratory Analyses on the Secondary Outcome: GCF Biomarker Levels.

| | Different in Obese and Control at Baseline (Table 1) | Different in Obese and Control during Treatment (Appendix Table 5) | Associated with Alignment Rate (Appendix Table 6) |
|---|---|---|---|
| Clinical indices | | | |
| Plaque index | No | No | – |
| Gingival index | No | No | – |
| uWMS | No | No | – |
| GCF volume | Yes | Yes | No |
| GCF biomarkers | | | |
| Adiponectin | No | No | – |
| Leptin | Yes | Yes | Yes |
| Resistin | Yes | Yes | Yes |
| MPO | Yes | Yes | Yes |
| CRP | No | No | – |
| MMP8 | Yes | Yes | No |
| MMP9 | No | No | – |
| TIMP1 | Yes | Yes | No |
| MMP8/TIMP1 | No | No | – |
| MMP9/TIMP1 | Yes | Yes | No |
| RANKL | Yes | Yes | Yes |

CRP, C-reactive protein; GCF, gingival crevicular fluid; MMP8, matrix metalloproteinase 8; MMP9, matrix metalloproteinase 9; MPO, myeloperoxidase; RANKL, receptor activator of nuclear factor kappa-B ligand; TIMP1, tissue inhibitor of metalloproteinase 1; uWMS, unstimulated whole-mouth salivary flow rate.

model that initial alignment can increase by 0.01 mm/d while overall alignment increases by 0.004 mm/d for every millimeter of initial irregularity (Woodhouse et al. 2015). However, significant differences were also found in the GCF biochemical profile between obese and normal-weight patients, and to our knowledge, this represents the first prospective data to suggest that obese patients may respond differently to those with normal weight during routine orthodontic treatment.

Appliance variation has little or no effect on rate of orthodontic tooth movement (Scott et al. 2008; Woodhouse et al. 2015). Interestingly, we found that obesity does influence tooth movement, as obese patients had increased movement rates compared with normal-weight patients. Statistical modeling of alignment rate and its change through time (see Table 2) demonstrated that obesity and initial irregularity at each phase explained part of the variation seen in alignment. Given the absence of a significant interaction between obesity and time, the difference in alignment rate between obese and normal-weight patients was consistently present through the alignment process and independent of confounders.

Importantly, the groups in this investigation were not different in terms of baseline demographics, including plaque/gingival indices and irregularity, with BMI representing the only significant difference. However, a number of differences existed in baseline GCF parameters between groups, including GCF flow rate and levels of several biomarkers. The proinflammatory adipokines leptin and resistin were both elevated in the GCF of obese patients (Suresh et al. 2016), suggestive of a baseline proinflammatory state within the periodontium of these individuals. It is also consistent with the significantly increased levels of MPO, an established marker for inflammation in the GCF (Marcaccini et al. 2010; Navarro-Palacios et al. 2014). Interestingly, the levels of several biochemical mediators of tissue remodeling were also increased at baseline in the obese group, including MMP8, TIMP1, MMP9/TIMP1, and RANKL, providing evidence of an altered inflammatory biochemical profile in the GCF of obese patients.

Among the GCF biomarkers assayed, leptin, resistin, MPO, and RANKL most predictably accounted for the observed differences in rate of tooth movement. The levels of these biomarkers differed significantly between obese and normal-weight patients both before and during treatment while being significantly associated with the amount of tooth movement observed. Previous studies have reported that orthodontic tooth movement is followed by a decrease in GCF leptin (Dilsiz et al. 2010) and an increase in both MPO (Marcaccini et al. 2010; Navarro-Palacios et al. 2014) and RANKL (Grant et al. 2013). Resistin, like leptin, is upregulated in inflamed gingival tissue compared with healthy tissue (Suresh et al. 2016), but the relationship between GCF resistin and orthodontic tooth movement has not been investigated previously. Variation in the levels of proinflammatory adipokines has been identified in the GCF of obese and normal-weight individuals with periodontal disease (Zimmermann et al. 2013; Gonçalves et al. 2015; Duzagac et al. 2016; Suresh et al. 2016), but data relating to adipokines during orthodontic tooth movement are sparse (Dilsiz et al. 2010).

The strengths of the present study include its prospective design (Papageorgiou, Xavier, et al. 2015), baseline comparability between experimental groups, absence of dropouts at completion, and use of measurement blinding. Moreover, obesity was defined according to widely accepted and reliable

international measures. Collectively, this means that the respective risk for selection, attrition, and detection bias is low. The study sample was based on a conservative a priori power calculation, and planned dropouts did not occur. However, some potential limitations include the fact that height and weight measurements were only taken at baseline and adiposity is not necessarily a static measure. Indeed, in an adolescent population, underlying growth might have influenced BMI during the course of the investigation, although with a mean observation of 158 d and mean patient age of 15.1 y, this effect may have been negligible. In addition, only BMI was used to classify adiposity, which can limit the identification of overweight and could have been reduced by adding estimates of adiposity (fat mass index) and fat distribution (waist-to-height ratio) (Bibiloni Mdel et al. 2013). Moreover, in a cohort undergoing routine orthodontic treatment with fixed appliances, it is not practical to see each patient at exactly the same time point for each adjustment or identify the absolute first time point that alignment is complete for every patient. For these reasons, the increased rates of tooth movement identified in the obese group may not have resulted in a clinically significant reduction in time to final alignment. In addition, while observed differences in rates of tooth movement are tangible effects with obvious potential clinical relevance, the underlying biological mechanisms are likely to be complex. The measured differences in GCF biomarkers may be associated with the interrelationship between obesity and tooth movement, but this study provides no conclusive evidence. Further investigation will be required to elucidate the precise role of each biomarker in mediating tooth movement. However, this investigation provides evidence that informs clinical practice in orthodontics and wider health care. The results are applicable to obese and normal-weight adolescent patients, although it should be remembered that adipose tissue can behave differently according to age group in other body systems (Palmer and Kirkland 2016). A proinflammatory obese state can influence orthodontic tooth movement, with significant associations between levels of specific biomarkers within the GCF of obese patients. These results highlight potential implications for orthodontic treatment in obese subjects, and one area for future research would be a comparison of postorthodontic stability.

This prospective clinical study investigated tooth alignment in obese and normal-weight patients undergoing fixed-appliance orthodontic treatment. Obese patients needed less time to achieve tooth alignment compared with normal-weight patients, but this was nonsignificant. After adjusting for confounders, rate of orthodontic tooth movement was significantly higher in obese patients compared with normal-weight patients. Explorative analyses indicated GCF levels of leptin, resistin, MPO, and RANKL were significantly different between obese and normal-weight patients and associated with observed rates of tooth movement.

## Author Contributions

H.F. Saloom, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript; S.N. Papageorgiou, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; G.H. Carpenter, M.T. Cobourne, contributed to conception, design, and data interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

## Acknowledgments

This work was funded by the Ministry of Higher Education and Scientific Research of Iraq through an educational scholarship to H.F.S. The authors gratefully acknowledge the help of Professor Fraser McDonald, Dr. Dirk Bister, and Dr. Anna Gibilaro in facilitating access to patients undergoing orthodontic treatment with fixed appliances at the King's College London Dental Institute, and Dr David Moyes for help with the Luminex assays. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

## References

Bibiloni Mdel M, Pons A, Tur JA. 2013. Defining body fatness in adolescents: a proposal of the AFAD-A classification. PLoS One. 8(2):e55849.

Bland JM, Altman DG. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1(8476):307–310.

Deng T, Lyon CJ, Bergin S, Caligiuri MA, Hsueh WA. 2016. Obesity, inflammation, and cancer. Annu Rev Pathol. 11:421–449.

Dilsiz A, Kilic N, Aydin T, Ates FN, Zihni M, Bulut C. 2010. Leptin levels in gingival crevicular fluid during orthodontic tooth movement. Angle Orthod. 80(3):504–508.

Duzagac E, Cifcibasi E, Erdem MG, Karabey V, Kasali K, Badur S, Cintan S. 2016. Is obesity associated with healing after non-surgical periodontal therapy? A local vs. systemic evaluation. J Periodontal Res. 51(5):604–612.

Gonçalves TE, Zimmermann GS, Figueiredo LC, Souza Mde C, da Cruz DF, Bastos MF, da Silva HD, Duarte PM. 2015. Local and serum levels of adipokines in patients with obesity after periodontal therapy: one-year follow-up. J Clin Periodontol. 42(5):431–439.

Grant M, Wilson J, Rock P, Chapple I. 2013. Induction of cytokines, MMP9, TIMPs, RANKL and OPG during orthodontic tooth movement. Eur J Orthod. 35(5):644–651.

Hotamisligil GS. 2006. Inflammation and metabolic disorders. Nature. 444(7121):860–867.

Issa RI, Griffin TM. 2012. Pathobiology of obesity and osteoarthritis: integrating biomechanics and inflammation. Pathobiol Aging Age Relat Dis. 2(2012):pii:17470.

Ivaska KK, Huovinen V, Soinio M, Hannukainen JC, Saunavaara V, Salminen P, Helmio M, Parkkola R, Nuutila P, Kiviranta R. 2016. Changes in bone metabolism after bariatric surgery by gastric bypass or sleeve gastrectomy. Bone. 95:47–54.

Kapoor P, Kharbanda OP, Monga N, Miglani R, Kapila S. 2014. Effect of orthodontic forces on cytokine and receptor levels in gingival crevicular fluid: a systematic review. Prog Orthod. 15:65.

Keller A, Rohde JF, Raymond K, Heitmann BL. 2015. Association between periodontal disease and overweight and obesity: a systematic review. J Periodontol. 86(6):766–776.

Lin LI. 1989. A concordance correlation coefficient to evaluate reproducibility. Biometrics. 45(1):255–268.

Little RM. 1975. The irregularity index: a quantitative score of mandibular anterior alignment. Am J Orthod. 68(5):554–563.

Loe H, Silness J. 1963. Periodontal disease in pregnancy: I. prevalence and severity. Acta Odontol Scand. 21:533–551.

López-Gómez JJ, Pérez Castrillón JL, de Luis Román DA. 2016. Impact of obesity on bone metabolism. Endocrinol Nutr. 63(10):551–559.

Marcaccini AM, Amato PA, Leão FV, Gerlach RF, Ferreira JT. 2010. Myeloperoxidase activity is increased in gingival crevicular fluid and whole saliva after fixed orthodontic appliance activation. Am J Orthod Dentofacial Orthop. 138(5):613–616.

Must A, Phillips SM, Tybor DJ, Lividini K, Hayes C. 2012. The association between childhood obesity and tooth eruption. Obesity (Silver Spring). 20(10):2070–2074.

Navarro-Palacios A, García-López E, Meza-Rios A, Armendariz-Borunda J, Sandoval-Rodriguez A. 2014. Myeloperoxidase enzymatic activity is increased in patients with different levels of dental crowding after initial orthodontic activation. Am J Orthod Dentofacial Orthop. 146(1):92–97.

Neeley WW II, Gonzales DA. 2007. Obesity in adolescence: implications in orthodontic treatment. Am J Orthod Dentofacial Orthop. 131(5):581–588.

Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera SF, et al. 2014. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the global burden of disease study 2013. Lancet. 384(9945):766–781.

Ouchi N, Parker JL, Lugus JJ, Walsh K. 2011. Adipokines in inflammation and metabolic disease. Nat Rev Immunol. 11(2):85–97.

Palmer AK, Kirkland JL. 2016. Aging and adipose tissue: potential interventions for diabetes and regenerative medicine. Exp Gerontol. 86:97–105.

Papageorgiou SN, Reichert C, Jäger A, Deschner J. 2015. Effect of overweight/obesity on response to periodontal treatment: systematic review and a meta-analysis. J Clin Periodontol. 42(3):247–261.

Papageorgiou SN, Xavier GM, Cobourne MT. 2015. Basic study design influences the results of orthodontic clinical investigations. J Clin Epidemiol. 68(12):1512–1522.

Pierpont YN, Dinh TP, Salas RE, Johnson EL, Wright TG, Robson MC, Payne WG. 2014. Obesity and surgical wound healing: a current review. ISRN Obes. 2014:638936.

Pradeep AR, Priyanka N, Prasad MV, Kalra N, Kumari M. 2012. Association of progranulin and high sensitivity CRP concentrations in gingival crevicular fluid and serum in chronic periodontitis subjects with and without obesity. Dis Markers. 33(4):207–213.

Salamat MR, Salamat AH, Janghorbani M. 2016. Association between obesity and bone mineral density by gender and menopausal status. Endocrinol Metab. 31(4): 547–558.

Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem. 270(45):26746–26749.

Schulz KF, Grimes DA. 2005. Sample size calculations in randomised trials: mandatory and mystical. Lancet. 365(9467):1348–1353.

Scott P, DiBiase AT, Sherriff M, Cobourne MT. 2008. Alignment efficiency of Damon3 self-ligating and conventional orthodontic bracket systems: a randomized clinical trial. Am J Orthod Dentofacial Orthop. 134(4):470.e1–8.

Silness J, Loe H. 1964. Periodontal disease in pregnancy: II. Correlation between oral hygiene and periodontal condition. Acta Odontol Scand. 22:121–135.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. 2001. The hormone resistin links obesity to diabetes. Nature. 409(6818):307–312.

Suresh S, Mahendra J, Singh G, Pradeep AR, Sundaravikram, Sekar H. 2016. Comparative analysis of GCF resistin levels in obese subjects with and without periodontal disease. J Clin Diagn Res. 10(5):ZC71– ZC74.

Suvan J, D'Aiuto F, Moles DR, Petrie A, Donos N. 2011. Association between overweight/obesity and periodontitis in adults: a systematic review. Obes Rev. 12(5):e381–e404.

von Bremen J, Lorenz N, Ruf S. 2016. Impact of body mass index on oral health during orthodontic treatment: an explorative pilot study. Eur J Orthod. 38(4):386–392.

von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP; STROBE Initiative. 2008. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. J Clin Epidemiol. 61(4):344–349.

Woodhouse NR, DiBiase AT, Johnson N, Slipper C, Grant J, Alsaleh M, Donaldson AN, Cobourne MT. 2015. Supplemental vibrational force during orthodontic alignment: a randomized trial. J Dent Res. 94(5):682–689.

World Health Organization/Royal College of Paediatrics and Child Health. 2016. GIRLS UK Body mass index (BMI) 2-20 years; BOYS UK Body mass index (BMI) 2-20 years. London (UK): RCPCH; [accessed 2017 Jan 9]. http://www.rcpch.ac.uk/system/files/protected/page/GIRLS%20and%20BOYS%20BMI%20CHART.pdf.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature. 372(6505):425–432.

Zimmermann GS, Bastos MF, Dias Goncalves TE, Chambrone L, Duarte PM. 2013. Local and circulating levels of adipocytokines in obese and normal weight individuals with chronic periodontitis. J Periodontol. 84(5):624–633.

Journal of Orthodontics

ISSN: 1465-3125 (Print) 1465-3133 (Online) Journal homepage: http://www.tandfonline.com/loi/yjor20

# Periodontal parameters of a self-ligating bracket

Hayder F. Saloom & Martyn T. Cobourne

**To cite this article:** Hayder F. Saloom & Martyn T. Cobourne (2016) Periodontal parameters of a self-ligating bracket, Journal of Orthodontics, 43:4, 253-254, DOI: 10.1080/14653125.2016.1240971

**To link to this article:** http://dx.doi.org/10.1080/14653125.2016.1240971

*Journal of Orthodontics*, Vol. 43, 2016, 253–254

# Periodontal parameters of a self-ligating bracket

The concept of a self-ligating bracket (SLB) is not a new one in orthodontics, but continued research and development in the design and manufacturing of these brackets has meant that a wide range of mechanically reliable SLBs are now commercially available. Although there have been many claims regarding the theoretical clinical advantages of these brackets to both the patient and orthodontist, much of the current clinical evidence suggests that they perform within similar parameters to conventional brackets. However, an undeniable difference between all SLBs and their conventional counterparts is the lack of an elastomeric to keep the archwire in place. This presents some obvious potential advantages, not least in terms of maintaining oral hygiene and promoting both gingival and periodontal health during treatment. However, the opening and closing mechanism associated with SLBs may itself have some impact on plaque retention, depending upon the design, but currently there is very little data relating to how SLBs perform in relation to these parameters.

There is certainly some diversity in the results of investigations that have compared the influence of SLBs and conventional brackets on plaque accumulation, gingival and periodontal health. However, two recently published systematic reviews have gone some way to showing that SLBs do not seem to perform any better than conventional brackets in terms of these variables (Arnold *et al*. 2016; Yang *et al*. 2016). The Arnold study carried out a systematic evaluation of adolescent populations over the short (4–6 weeks) and slightly longer-term (3–6 months) and showed only 'scarce' evidence of a greater plaque index for conventional brackets at 3–6 months of treatment. However, gingival index and pocket depth pooled estimates revealed no significant differences between SLBs and conventional brackets at either time-point (Arnold *et al*. 2016). The Yang study just compared plaque indices associated with passive SLBs and conventional brackets and found no significant differences. This data was derived from four studies with high heterogeneity and in the case of two of them, very wide confidence intervals and therefore should be treated with some caution (Yang *et al*. 2016). However, the best current evidence would suggest that SLBs should not be offered to patients on the basis that they help to keep the teeth cleaner and healthier, but as is the case in much orthodontic research, this best current evidence could be of better quality.

In this issue of the *Journal of Orthodontics* Bergamo and co-workers have investigated gingival crevicular fluid (GCF) volume and a number of periodontal parameters in a prospective cohort of subjects bonded with different bracket types in the upper labial segment (Bergamo *et al*. 2016). Interestingly, they find significant differences in plaque index and GCF volume associated with SmartClip brackets at 60 days following appliance placement. This suggests that other variables, such as GCF volume may be influenced by bracket design and should be followed up with more definitive prospective data. Some attention should be given to the detail of the methodology. The GCF was collected by inserting paper strips into the gingival sulcus until resistance was felt and this method can increase local trauma, potentially affecting GCF volume (Chapple *et al*. 1996). However, these points notwithstanding, this investigation suggests a need for more data in relation to this subject. Indeed, the assay of GCF provides a relatively non-invasive means of evaluating multiple changes within the periodontium at the biochemical level and it would seem appropriate to extend some of these studies into this area of bracket-based research. There is some low-level evidence that levels of the substance P neuropeptide are lower in the GCF of teeth bonded with Damon SLBs in comparison to conventional over the short-term (Yamaguchi *et al*. 2009) although this does not seem to correlate with any differences in pain perception at the clinical level (Yang *et al*. 2016).

This is inevitably an area of great potential complexity and whilst variation in isolated biological and biochemical parameters associated with SLBs and conventional brackets are likely to be found, whether this has a fundamental influence on clinical performance is more difficult to predict. Certainly, the evidence to date would suggest that for virtually all the clinical variables that have been


DOI:10.1080/14653125.2016.1240971

tested, an orthodontic bracket will perform in a similar manner whether it is a SLB or not.

Hayder F. Saloom and Martyn T. Cobourne
King's College London
*martyn.cobourne@kcl.ac.uk*

## References

Arnold S, Koletsi D, Patcas R, Eliades T. The effect of bracket ligation on the periodontal status of adolescents undergoing orthodontic treatment. A systematic review and meta-analysis. J Dent. 2016 (in press).

Bergamo AZN, Nelson-Filho P, Romano FL, da Silva RAB, Saraiva MCP, Matsumoto da Silva LAB, Matsumoto MAN. Gingival crevicular fluid volume and periodontal parameters alterations after use of conventional and self-ligating brackets. J Orthod. 2016; 43: 260–267. doi:10.1080/14653125.2016.1221214

Chapple IL, Socransky SS, Dibart S, Glenwrigh DH, Matthews JB. Chemiluminescent assay of alkaline phosphatase in human gingival crevicular fluid: investigations with an experimental gingivitis model and studies on the source of the enzyme within crevicular fluid. J Clin Periodontol. 1996; 23: 587–594.

Yamaguchi M, Takizawa T, Nakajima R, Imamura R, Kasai K. The Damon system and release of substance p in gingival crevicular fluid during orthodontic tooth movement in adults. *World J Orthod* 2009; 10: 141–146.

Yang X, Su N, Shi Z, Xiang Z, He Y, Han X, Bai D. Effects of self-ligating brackets on oral hygiene and discomfort: A systematic review and meta-analysis of randomized controlled clinical trials. Int J Dent Hyg. 2016 (in press).